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Degree: Master of Science

Year this Degree Granted: 2003

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University of Alberta

ROLE OF PROTEIN PHOSPHATASES IN INHIBITION OF SYMPATHETIC NEURON APOPTOSIS BY CERAMIDE

by

Gregory Joseph Plummer

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Department of Pharmacology

Edmonton, Alberta
Spring 2003

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Role of Protein Phosphatases in Inhibition of Sympathetic Neuron Apoptosis by Ceramide submitted by Gregory Joseph Plummer in partial fulfillment of the requirements for the degree of Master of Science.

To my parents, family and friends, who gave me their unconditional support, for that I am forever thankful.

ABSTRACT

We examined the anti-apoptotic effect of ceramide in rat sympathetic neurons deprived of NGF. Upon NGF withdrawal cultured sympathetic neurons undergo apoptosis (Deshmukh and Johnson, 1997). However, addition of ceramide prevents the appearance of classical trademarks of apoptosis such as nuclear fragmentation and caspase -3 activation (Nair et al. 2000; Song and Posse de Chaves, 2002). Here, we show that treatment with C_6-ceramide or *threo*-PPMP, an inhibitor of ceramide conversion to glucosylceramide also prevent loss of mitochondrial potential, a late hallmark in sympathetic neuron apoptosis.

We investigated the role of serine/threonine protein phosphatases as targets for ceramide action. We demonstrate that ceramide activates PP-1 and that PP1-activation is essential for C_6-ceramide anti-apoptotic activity. Additionally, we examined the phosphorylation status of pRb, a substrate of PP-1 implicated in neuronal apoptosis. We found that pRb is hyperphosphorylated upon NGF-deprivation and that ceramide can prevent such hyperphosphorylation by a mechanism dependent on PP-1 activation.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor, Dr. Elena Posse de Chaves for her support and guidance throughout my graduate career. I am especially thankful for the knowledge that I have gained from her in all aspects of scientific endeavors, both in experimental techniques and an advancement of critical thinking. I am also very grateful for the financial support that she provided for me.

I would also like to thank all of the members of my supervisory committee, including Dr. Elena Posse de Chaves, Dr. Peter Smith and Dr. Robert Campenot for their helpful suggestions and constructive criticism, and for the tremendous feedback helping me to advance my oral, written, and visual style. Additionally. I would also like to thank Dr. Charles Holmes for the many informative discussions we shared and for the providing me with unlimited access to his laboratory resources and staff to complete the work involving phosphatase analysis.

I am extremely grateful to the all members of the Posse de Chaves laboratory (2000-2003) for the day-to-day support they have given me. I would like to thank our wonderful technical team Barbara Zielnik and Valeria Mancinelli and our previous technician Karen Lund for all the hard work that goes into keeping a research lab going on a daily basis. Also I would like to thank the other

graduate student Mee-Sook Song for very informative discussions and good friendship over the past three years.

Lastly, I would like to thank all of the graduate students in the pharmacology department who I have known over the years for their friendship and support.

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LIST OF ABBREVIATIONS

CAPP, ceramide activated protein phosphatase

CDK, Cyclin dependent kinase

DHCer, dihydroceramide

I-2, Inhibitor-2

NGF, nerve growth factor

OA, Okadaic Acid

PA, Phosphatidic Acid

PPMP, dl-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol

pRb, retinoblastoma gene product

PP-1, protein phosphatase type-1

PP2A, protein phosphatase type 2A

SM, Sphingomyelin

SMase, Sphingomyelinase

1) INTRODUCTION

The study of the metabolism of glycerophospholipids has revealed an important role for lipids as signaling molecules and second messengers in intracellular signaling cascades highlighted by the important functions of diacylglycerol (DAG), inositol trisphosphate (IP3), arachidonate, eicosanoids, and platelet activating factor. Over the last two decades, similar investigations of the metabolism of sphingolipids have brought to the forefront the role of sphingolipid metabolites as important signaling molecules. Sphingolipids are involved in cell contact responses, and are structural components of receptors, protein anchors, and function as markers of cell differentiation and tumour progression. Sphingolipid metabolism is a highly regulated process, which controls the levels of individual lipids, as well as inter-conversion between them. At the hub of sphingolipid metabolism is ceramide, which has been recognized as an important signaling molecule. In particular, ceramide serves as a second messenger in cellular responses to very diverse agonists including cytokines, radiation, Vitamin D_3 and neurotrophins (Hannun, 1996).

1.1 CERAMIDE

1.1.1 Physical and structural properties of ceramide

Ceramide consists of an 18-carbon sphinganine backbone, linked to a variable fatty acid chain consisting of 2-28 carbons (Fig.1). Due its extreme hydrophobicity, even in comparison with phospholipids, ceramide is present exclusively in cellular membranes, and there is no "cytosolic" or "free ceramide", (reviewed in Kolesnick et al., 2000). The most abundant isomer of natural ceramide is the D-*erythro*-C_{18}-ceramide, which also has the greatest bioactivity. The extreme hydrophobic nature of ceramide requires targets of ceramide to be either transiently or permanently associated with cellular membranes.

1.1.2 Biological effects of ceramide

Ceramide is involved in diverse cellular processes including differentiation, proliferation, senescence, cell cycle arrest, survival and apoptosis. Ceramide was first implicated as a signaling intermediate in differentiation of HL-60 cells. Okazaki *et al.* showed that treatment of HL-60 cells with 1α, 25-dihydroxyvitamin D_3 activated a neutral sphingomyelinase (SMase) inducing accumulation of ceramide and differentiation of these cells (Okazaki et al., 1989). The role of ceramide in HL-60 cell differentiation was confirmed with C_2-ceramide, which mimicked the effect of Vitamin D_3 (Okazaki et al., 1990). In addition, ceramide

also inhibited growth in HL-60 cells (Bielawska et al., 1992) and proliferation in *S.cerevisaie* (Fishbein et al., 1993) and Molt-4 cells (Jayadev et al., 1995). Treatment of Molt-4 cells with C_6-ceramide induced dephosphorylation of the retinoblastoma gene product (pRb) and arrested cells in the G_0/G_1 phase of the cell cycle (Dbaibo et al., 1995). Additionally pRb phosphorylation and protein kinase Cα (PKCα) activation was inhibited in molt-4 cells treated with C_2-ceramide (Lee et al., 1996a). The effect of ceramide on growth arrest is the result of dephosphorylation of pRb by activation of protein phosphatase-1 (PP-1) (Kishikawa et al., 1999). Ceramide also induces apoptosis in many cells. However, the effect of growth arrest and apoptosis caused by ceramide are independent (Hannun, 1996). In reference to apoptosis, ceramide was first shown to induce apoptosis in human myeloid leukemia cells. Treatment of U937 and HL-60 cells with C_2-ceramide or SMase caused DNA laddering, a hallmark of apoptotic cell death (Obeid et al., 1993), (Jarvis et al., 1994). The roles of ceramide as an inducer of apoptosis have also been demonstrated in Jurkat T cells (Dbaibo et al., 1993) and many other cell types (Hannun and Luberto, 2000). The finding by Smyth et al. that ceramide activated the protease prICE, inducing poly (ADP-ribose) polymerase cleavage (PARP) (Smyth et al., 1996), and that apoptosis induced by ceramide is blocked by Bcl-2 (Zhang et al., 1996) demonstrated the direct involvement of ceramide with regulators of apoptosis.

The production of ceramide in cells is the result of two major pathways 1) *de novo* synthesis of ceramide from palmitoyl-CoA and serine 2) hydrolysis of membrane sphingomyelin (SM) by sphingomyelinases (SMases).

1.2 CERAMIDE METABOLISM

1.2.1 Generation of ceramide by *de novo synthesis*

The initial step in ceramide production via the *de novo* pathway involves the condensation of L-serine with palmitoyl-CoA by the enzyme serine palmitoyltransferase (SPT) (Fig 2). Using highly purified preparations of Golgi and endoplasmic reticulum from mouse liver, Mandon *et al.*, demonstrated that this reaction occurs at the cytosolic side of the endoplasmic reticulum (Mandon et al., 1992). The action of serine palmitoyltransferase yields 3-ketodihydrosphinganine, which is rapidly reduced to sphinganine. Conversion of 3-ketosphinganine to sphinganine is catalyzed by the action of the NADPH-dependent 3-ketosphinganine reductase (Mandon et al., 1992). Following this step the amino group of sphinganine is acylated by sphinganine N-acyltransferase (also referred to as ceramide synthase) to form dihydroceramide (Merrill and Wang, 1986). The high activity of ceramide synthase prevents accumulation of free sphingoid bases (Merrill and Wang, 1986), which can be cytotoxic (Merrill, 1983). Ceramide synthase is inhibited by the fungal toxin Fumonisin B1 (FB1) (Wang et al., 1991), which shares structural similarities with sphinganine. Dihydroceramide is converted to ceramide by the insertion of a trans double bond at the 4,5 position of the sphinganine backbone. This reaction

is catalyzed by dihydroceramide desaturase (Rother et al., 1992), (Merrill and Wang, 1986). The double bond is the only structural variation between dihydroceramide and ceramide. This structural change accounts for a significant difference in biological activity, as D-*erythro*-dihydroceramide is inactive (Bielawska et al., 1993). Inhibitors of the ceramide biosynthetic pathway have been identified. ISP-1/myriocin an immunosuppressant homologous to sphingosine inhibits serine palmitoyltransferase at picomolar concentrations (Miyake et al., 1995).

1.2.2 Conversion of ceramide to SM and glycosphingolipids

Although ceramide is a bioactive metabolite, it is also an intermediate in sphingolipid synthesis. Once synthesized, ceramide is rapidly converted to SM or glycosphingolipids. SM is localized mainly at the outer leaflet of the plasma membrane and the membranes of related organelles such as endocytotic vesicles and lysosomes (reviewed in Merrill and Jones, 1990). Conversion of ceramide to SM takes place at the lumenal face of the Golgi apparatus (Lipsky and Pagano, 1983).

Synthesis of glycosphingolipids from ceramide occurs at the cytosolic face of the Golgi and in pre-Golgi (ERGIC) compartments (Futerman and Pagano, 1991). The initial step in most glycosphingolipid assembly involves conversion of ceramide to glucosylceramide by glucose transfer from UDP-glucose to ceramide by glucosylceramide synthase. Inhibition of glucosylceramide synthase has been

exploited to elevate levels of *de novo* synthesized ceramide. 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) a potent inhibitor of glucosylceramide synthase was synthesized by Radin and colleagues (Vunnam and Radin, 1980), the D-*threo* isomer of PDMP has the greatest inhibitory effect (Inokuchi and Radin, 1987), and the *erythro* isomer is inactive in enzyme inhibition. Recently the analogues 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and 1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol (PPPP/P4) have been identified as more potent inhibitors of glucosylceramide synthase (Radin et al., 1993), (Abe et al., 1995). Use of inhibitors of glucosylceramide synthase has demonstrated a potential therapeutic role for ceramide (Radin, 2001).

Several steps in the synthesis of ceramide and glycosphingolipids have been demonstrated to be regulated by drugs that are currently in use as therapeutic agents (Fig. 2) (Senchenkov et al., 2001), highlighting the importance of these pathways as pharmacological targets.

1.2.3 Generation of ceramide by SM hydrolysis

Ceramide is also generated from the hydrolysis of SM (Fig.3). The enzymes that catalyze this reaction are sphingomyelinases (SMases) which are special isoforms of Phospholipase C (PLC) directed towards SM. The action of SMases produces ceramide and phosphocholine. There are at least 5 main

classes of SMases distinguished by the optimum pH of action, cation requirements, and cellular location. The action of SMases has been reviewed in detail elsewhere (Levade and Jaffrezou, 1999, Hannun et al., 2001). Activation of SMases in response to extracellular signals was originally viewed as the main pathway for generation of bioactive ceramide. This mechanism is often referred to as the "Sphingomyelin cycle" (Hannun, 1994). Currently, the evidence supports the idea that both pathways of ceramide generation are equally important in ceramide signaling.

1.3 TOOLS TO STUDY CERAMIDE ACTION

Long-chain ceramides cannot be easily delivered in aqueous solutions (i.e. media), due to the insolubility of natural ceramides. Therefore, short-chain analogues of ceramide (C_2-ceramide or C_6-ceramide) have been used extensively to study the cellular effects of ceramide. Short-chain ceramides are still hydrophobic but once dissolved in organic solvents, "swell" in aqueous solutions forming micelles. Like long-chain ceramides synthetic short chain ceramides also partition into membranes (Simon and Gear, 1998). Short chain ceramides seem to be endocytosed into endosomes and targeted to Golgi (Babia et al., 2001). Use of short chain ceramides, and the information they provide has been criticized in the past, however there are several points, which validate this approach. 1) C_2-ceramide occurs naturally in mammalian cells (Abe et al., 1996),

(Lee et al., 1996b; Song and Posse de Chaves, 2002). 2) Short-chain ceramides mimic the responses of stress-inducers (i.e. FAS, TNFα). In addition, the availability of the biologically inactive C_2- and C_6-dihyrdroceramide provides an excellent negative control for short chain ceramides. The benefits of using these short chain analogues have been analyzed recently (Ghidoni et al., 1999). In addition to using short chain ceramide analogues in studies on ceramide action, an alternative approach is to manipulate the *de novo* pathway of sphingolipid synthesis to elevate levels of cellular ceramide. The use of PPMP (described in 1.2.2) has been established as an effective tool to increase cellular ceramide. In many cases, it has been shown that the effect of short chain ceramide mimics the effect of natural ceramide accumulated through manipulation of biosynthetic pathways (de Chaves et al., 1997).

1.4 TARGETS OF CERAMIDE

Although ceramide has a role as an effector in a vast number of processes, only a small number of targets have been identified as being directly regulated by ceramide. These targets include ceramide activated protein kinase (CAPK), which has been identified as the kinase suppressor of ras (KSR) (Zhang et al., 1997), cathepsin D (Heinrich et al., 2000), atypical protein kinase Cζ (PKCζ) (Lozano et al., 1994), and ceramide activated protein phosphatases (CAPPs) (Dobrowsky and Hannun, 1992; (Chalfant et al., 1999). However, as it

pertains to this thesis, the effect of ceramide activated protein phosphatases will be the only target extensively discussed.

1.4.1 Serine/Threonine protein phosphatases

Ceramide activates phosphatases belonging to the serine/threonine (ser/thr) directed families of phosphatases. The two major families of ser/thr protein phosphatases are type –1 (PP-1) and type 2 (PP2). The PP2 family can be further sub-classified into type-2A (PP2A), type 2B (PP2B or calcineurin) and type 2C (PP2C), based on the divalent cation requirements and inhibitor sensitivities. Relevant to our studies are PP-1 and PP2A.

Protein phosphatase-1 (PP-1)

PP-1 is expressed in all eukaryotic tissues and is involved in many diverse cellular processes such as glycogen metabolism, muscle contraction, RNA splicing, neuronal activities including synaptic plasticity and ion channel modulation (reviewed recently in Cohen, 2002; Barford et al., 1998). Interestingly Foulkes and Maller demonstrated a role for PP-1 in cell cycle regulation (Foulkes and Maller, 1982). In vivo the PP-1 holoenzyme exists as a heterodimer consisting of a catalytic and a regulatory subunit. The catalytic sub-unit of PP-1 (PP-1C) is the result of the expression of three genes *PP1Cα*, *PP1Cβ*, and *PP1Cγ*. The gene products formed from these genes are PP1Cα, PP1Cβ (previously referred to as PP1Cδ), and PP1Cγ1/PP1Cγ2 respectively (Cohen,

1989). Free catalytic subunits of PP-1 are not present in tissue extracts suggesting that *in vivo* PP-1C is always complexed to a regulatory subunit. Multiple regulatory subunits have been identified for PP-1 (Table 1). In general, regulatory subunits of PP-1C have low peptide homology. However all subunits contain a PP-1C targeting sequence (Egloff et al., 1997). The first characterized native complex of PP-1 was PP-1C bound to inhibitor-2 (I-2) (Ballou et al., 1983). Binding of PP-1C to a given regulatory sub-unit targets the catalytic sub-unit to distinct cellular compartments and confers substrate specificity (Hubbard and Cohen, 1993). Regulatory subunits of PP-1 belong to different categories. Some subunits bind to PP-1C and target the catalytic subunit to specific substrates, while some regulatory subunits bind PP-1 and are substrates of PP-1 and are dephosphorylated by the enzyme. Binding of regulatory subunits seems to be mutually exclusive, allowing only single subunit binding to PP-1C. In addition, PP-1C activity is regulated by endogenous and exogenous inhibitors and by phosphorylation.

Inhibitors of PP-1

PP-1 is inhibited by a variety of substance or compounds including mammalian proteins, acidic phospholipids, and natural toxins. PP-1 inhibitors that occur in mammalian tissues include two heat stable proteins termed inhibitor-1 (I-1) and inhibitor-2 (I-2) and the phosphoprotein DARPP-32 (Huang and Glinsmann, 1976; Nimmo and Cohen, 1978; Hemmings et al., 1984). I-1 and

I-2 are potent inhibitors of PP-1 and do not inhibit PP2A activity (Cohen, 1989). This specificity has been used to differentiate protein phosphatase family members, as only phosphatases sensitive to I-1 and I-2 are classified as type-1 (PP-1) (Nimmo and Cohen, 1978).

PP-1C is also inhibited by a variety of natural toxins. Okadaic acid (OA), a marine sponge toxin responsible for diarrhetic shellfish poisoning was one of the first natural compounds shown to inhibit protein phosphatases. It inhibits PP-1 at nanomolar concentrations (Bialojan and Takai, 1988), but is a more potent inhibitor of PP2A (see below). In addition, microcystin a hepatotoxin produced by blue green algae inhibits PP-1 and PP2A in a manner similar to OA (MacKintosh et al., 1990). PP-1C is also inhibited by acidic phospholipids. Phosphatidic acid (PA) inhibits PP1Cα and PP1Cγ in vitro (Chalfant et al., 1999), (Jones and Hannun, 2002).

In addition to endogenous and exogenous inhibitory molecules, PP-1 activity can also be regulated by inhibitory phosphorylation on C-terminal threonines. This phosphorylation is catalyzed by cyclin-dependent kinase 2 (CDK2) (Dohadwala et al., 1994). This Inhibitory phosphorylation of PP-1C by CDKs ensures that phosphatase activity corresponds to proliferative signals during the phases of the cell cycle (Liu et al., 1999).

Protein phosphatase 2A (PP2A)

PP2A is involved in regulation of metabolism, cell signaling, cell cycle, and telomerase activity (Barford et al., 1998). Like PP-1, PP2A exists in vivo as a multimeric complex. PP2A is a heterotrimeric holoenzyme consisting of a structural (A), regulatory (B), and catalytic subunit (C). As for PP-1, multiple regulatory subunits (Bα, Bβ, and Bγ) have been identified that regulate substrate specificity (McCluskey et al., 2002). These sub units are categorized in Table.2. PP2A is also inhibited by a variety of inhibitors. OA will inhibit both PP-1 and PP2A but PP2A is extremely sensitive to OA. OA completely inhibits PP2A at concentrations of 0.2-1.0nM *in vitro* (Dawson and Holmes, 1999; Bialojan and Takai, 1988). In contrast, PP-1 requires a concentration of OA one hundred fold greater for inhibition (PP-1 $IC50_{OA}$=20nM) (Dawson and Holmes, 1999). This differential sensitivity of PP2A and PP-1 to OA has classically been used to differentiate the two phosphatase activities.

1.4.2 Ceramide activated protein phosphatases

The first evidence for ceramide activating protein phosphatases came from rat T9 glioma cells in which C_2-ceramide activated a serine/threonine directed protein phosphatase (Dobrowsky and Hannun, 1992). Activation of a CAPP in *S.cervisiae* with biochemical characteristics similar to mammalian CAPP, demonstrated a role for CAPP in growth arrest (Fishbein et al., 1993). Sensitivity of mammalian and yeast CAPP to low concentrations of OA (IC_{50} 0.5-

1.0 nM), cation independence and resistance to the inhibitory actions of inhibitior-2, (Fishbein et al., 1993; Dobrowsky et al., 1993) demonstrated that this CAPP shared many biochemical properties with the type-2A (PP2A) serine/threonine phosphatases. In addition, C_2- and C_{18}- ceramide activate the catalytic (PP2AC) subunit, and the dimeric (AC) and trimeric ($AB_\alpha C$) PP2A complexes *in vitro* (Dobrowsky et al., 1993; Law and Rossie, 1995; Dobrowsky and Hannun, 1993; Galadari et al., 1998). Activation of CAPP/PP2A by ceramide causes downregulation of the proto-oncogene c-myc in HL-60 leukemia cells (Wolff et al., 1994), inactivation of PKCα in Molt-4 cells (Lee et al., 1996a), and dephosphorylation of the anti-apoptotic protein Bcl-2 in HL-60 cells (Ruvolo et al., 1999). More recently, PP-1 has also been reported as a target for ceramide. Short and long chain ceramides activate PP1Cα and PP1Cγ *in vitro* (Chalfant et al., 1999; Kishikawa et al., 1999). Activation of the catalytic subunits of PP-1 and PP2A, and the trimeric forms of PP2A (AB'C) by ceramide shows stereospecificity with D-*erythro*-C_6 and C_{18} ceramide being the most potent activators of PP1Cα, PP1Cγ, PP2AC, and trimeric PP2A (AB'C) (Dobrowsky and Hannun, 1993; Chalfant et al., 1999; Kishikawa et al., 1999). The differences in activation of PP-1 and PP2A by these isomers suggest ceramide interacts directly with the catalytic subunits of ser/thr phosphatases, and this interaction requires a specific orientation.

Downstream substrates of PP-1 affected by ceramide

In Molt-4 cells, C_6-ceramide activates PP-1 leading to dephosphorylation of the pRb and causing cell cycle arrest (Kishikawa et al., 1999; Dbaibo et al., 1995) (for more details concerning the action of PP-1 on pRb leading to growth arrest consult appendix A). In addition to activation of PP-1 by exogenous ceramide, elevated levels of cellular ceramide activate PP-1. Treatment of Jurkat leukemia cells and A549 adenocarcinoma cells with FAS ligand elevate ceramide levels leading to PP-1 dependent dephosphorylation of the mRNA modulating family of SR proteins (Chalfant et al., 2001) and decreased levels of the anti-apoptotic splice variants of caspase 9 and Bcl-x in A549 cells (Chalfant et al., 2002). Interestingly these studies suggest the requirement of endogenous ceramide for PP-1 activation (Chalfant et al., 2001).

1.5 CERAMIDE SIGNALING IN NEURONS

1.5.1 Effects of ceramide in cultured neurons

The initial evidence for ceramide signaling in neuronal cultures was demonstrated when Harel and Futerman showed that inhibition of sphingolipid synthesis by FB_1 prevented axon outgrowth in hippocampal neurons which could be reversed by treatment with ceramide (Harel and Futerman, 1993). Schwarz *et*

al. also demonstrated that inhibition of the conversion of ceramide to glucosylceramide could increase axonal branching in hippocampal neurons (Schwarz et al., 1995). Additionally, ceramide has been demonstrated as a second messenger for the p75 neurotrophin receptor. Dowbrowsky *et al.* demonstrated that nerve growth factor (NGF) could bind the $p75^{NTR}$ and activate SMase to generate ceramide in T9 glioma cells (Dobrowsky et al., 1994). In addition, others have shown that neurotrophins can bind $p75^{NTR}$ to accumulate ceramide (Casaccia-Bonnefil et al., 1996; Dobrowsky et al., 1995).

Ceramide can induce or protect from apoptosis in cultured neurons. Goodman and Mattson demonstrated that C_2-ceramide protected hippocampal neurons from apoptosis induced by excitotoxicity, oxidative stress, and β-amyloid, through activation of the transcription factor NFκB (Goodman and Mattson, 1996). In addition, activation of the $p75^{NTR}$ results in ceramide accumulation and neurite outgrowth in freshly cultured hippocampal neurons (Brann et al., 1999), meanwhile in older cultures; ceramide accumulation induces apoptosis (Brann et al., 2002). In sensory neurons short chain ceramide analogues inhibits apoptosis in the absence of neurotrophins and enhances survival of these neurons in the presence of neurotrophic factors (Ping and Barrett, 1998). In contrast, short chain ceramide analogues induce apoptosis in mesencephalic neurons (Brugg et al., 1996). In sympathetic neurons, ceramide inhibits axonal growth in the presence of NGF, and inhibits apoptosis in NGF-deprived neurons (discussed in 1.5.3).

1.5.2 Sympathetic neurons and apoptosis

Apoptosis is an essential process during development and tissue remodeling. During development of the nervous system, more than 50% of neurons undergo apoptosis as a result of competition for limited amounts of target released neurotrophic factors (reviewed in Oppenheim, 1991). Sympathetic neurons depend on NGF for survival *in vivo* and *in vitro* (Levi-Montalcini, 1987). NGF binds to two receptors a tyrosine kinase receptor TrkA and the p75 neurotrophin receptor $p75^{NTR}$. NGF binds to TrkA receptors in the distal axons and the NGF-TrkA complex is retrogradely transported to the cell bodies (reviewed in Miller and Kaplan, 2001). Newer evidence suggests that NGF is not transported to the cell body, however the survival signal remains intact (MacInnis and Campenot, 2002). After NGF-withdrawal, cultured sympathetic neurons undergo apoptosis in vitro. The process of apoptosis in sympathetic neurons has been extensively characterized (reviewed in Edwards and Tolkovsky, 1994; Deshmukh and Johnson, 1997; Chang et al., 2002). Apoptosis in these neurons requires protein synthesis (Martin et al., 1988). Morphological changes do not occur until 18-20h following NGF withdrawal. One of the earliest events in apoptosis caused by NGF- withdrawal is the induction of reactive oxygenase species (Greenlund et al., 1995). This is followed by increased activity of the transcription factor c-jun, loss of mitochondrial membrane potential and release of cytochrome c. As a result, death effector

proteases or caspases are activated (Deshmukh et al., 1996). In addition, NGF deprivation also causes changes in expression of cell cycle regulatory proteins (Freeman et al., 1994). This increase in regulators of the cell cycle prompted the hypothesis that deregulation of cell cycle control is linked to apoptosis, commonly referred to as the cell cycle hypothesis (Heintz, 1993). The cell cycle and apoptotic pathways share common intermediates including the p53 tumour suppressor. Involvement of cell cycle regulatory molecules was further implicated in apoptosis after NGF-withdrawal when Farnelli and Greene showed that neuronally differentiated PC12 cells and sympathetic neurons could be protected by cell cycle blockers acting at the G1/S transition (Farinelli and Greene, 1996). Subsequently, the involvement of cell cycle regulators was stressed by Park *et al.* showing that inhibitors of cyclin-dependent kinases protected sympathetic neurons from apoptosis induced by NGF-deprivation (Park et al., 1996). Activation of CDKs results in hyperphosphorylation of pRb (Weinberg, 1995). Therefore, one consequence of NGF-deprivation may be the hyperphosphorylation of pRb.

1.5.3 Ceramide and sympathetic neurons

Ceramide has two effects in sympathetic neurons, inhibition of axonal growth in the presence of NGF (de Chaves et al., 1997) and inhibition of the apoptotic pathway that follows NGF-deprivation (Ito and Horigome, 1995; Nair et al., 2000; de Chaves et al., 2001). Work by Elena Posse de Chaves *et al* showed

that elevation of ceramide in sympathetic neurons by treatment with C_6-ceramide or elevation of endogenous ceramide by *threo*-PPMP inhibited axonal growth in the presence of NGF (de Chaves et al., 1997). Using a compartmented culture system for sympathetic neurons developed by Robert Campenot, (Campenot, 1979) they demonstrated that the effect of ceramide on axonal elongation was localized to the distal axons (de Chaves et al., 1997) and partly due to inhibition of NGF uptake (de Chaves et al., 2001). In their studies however, they did not observe any effect of ceramide on survival in the presence of NGF. Ito & Horigome first suggested that ceramide might delay apoptosis in sympathetic neurons undergoing NGF-deprivation showing that C_2 ceramide and SMase treatment prevented cell death (Ito and Horigome, 1995). Nair *et al.* proposed a mechanism by which ceramide protects sympathetic neurons from apoptosis. Their studies suggest that ceramide inhibits the generation of reactive oxygen species (ROS) and c-jun activation (Nair et al., 2000). However, studies from our lab (Song and Posse de Chaves, 2002) suggest ceramide activates multiple pathways to protect sympathetic neurons from apoptosis.

The two major goals of this project are to further characterize the anti-apoptotic effect of ceramide in NGF-deprived sympathetic neurons and to investigate the mechanism(s) involved in the anti-apoptotic effect of ceramide in these neurons.

2) EXPERIMENTAL PROCEDURES

2.1 ***Materials*** – Leibovitz L15-CO_2 culture medium was purchased from Life Technologies, Inc. Rat serum was prepared from adult rat blood supplied by Health Science Lab Animal Services, University of Alberta. Trypsin and collagenase were obtained from Sigma. Rat-tail collagen was isolated as described (Hawrot and Patterson, 1979). Mouse nerve growth factor (2.5S) was purchased from Alomone Laboratories Ltd. (Jerusalem, Israel). Anti-NGF antibody (α-NGF) was purchased from Cedarlane Labs (Hornby, ON). C_6-ceramide, C_6-dihyroceramide, *threo-* (DL-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol) PPMP, and *erythro*-PPMP were obtained from Matreya Inc. (Pleasant Gap, PA). Okadaic Acid and Inhibitor-2 were generously provided by Dr. CF Holmes, (University of Alberta). MitoTracker® Orange (CM-H_2-TMRos) and MitoTracker® Green (Green FM) were purchased from Molecular probes (Eugene, OR). Polyclonal anti-Rb antibody and Molt-4 pRb control lysate was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Monoclonal anti- α/ β- tubulin, and phosphatidic acid (1,2-Di [9cis-9-octadecenoyl]-sn-glycero-3-phosphate) were purchased from Sigma. Monoclonal and polyclonal anti-IgGs were purchased from Pierce Biotechnology (Rockford, IL). All other materials where purchased from Sigma or Fisher Scientific.

2.2 *Culture of sympathetic neurons* - Sympathetic neurons were isolated from superior cervical ganglia of newborn Harlan Sprague-Dawley rats (Health Science Lab Animal Services, University of Alberta). Animal dissections were performed in a dissecting hood under a NIKON SMZ660 zoom stereo dissecting microscope. Ganglia were removed from the animal with sterile forceps and placed into plating medium. Isolated ganglia were washed once with sterile phosphate buffered saline and incubated in 1ml 1% (w/v) collagenase for 25 min at 37°C. Following collagenase treatment 100µl of 0.1% (w/v) trypsin was added for 5 min at 37°C. The ganglia were centrifuged for 2 min at 1,200 rpm. Supernatant was aspirated and ganglia were washed once in sterile phosphate buffered saline, and twice in plating medium containing rat serum. Each wash was followed by centrifugation at 1,200 rpm. Following the last centrifugation, ganglia were re-suspended in 800µl of plating medium and mechanically dissociated using flame-polished Pasteur pipets. Ganglia were successively titurated 10-15 times through pipets of decreasing diameter to obtain single cell preparations. The titurated ganglia suspension was then filtered through a sterile 40µm nylon cell strainer (Becton-Dickinson) and this solution was centrifuged for 3 min at 1,500 rpm. The supernatant was then aspirated and neurons were re-suspended in the desired amount of standard culture medium. Standard culture medium consisted of L15-CO_2 supplemented with the prescribed additives and 0.4 % methyl – cellulose, 2.5% rat serum, 1 mg/ml ascorbic acid and 50 ng/ml NGF. Neurons were plated on collagen coated 24-well plates at a density of 1 ganglion/well for immunoblot analysis and phosphatase activity assays or on 96-

well plates at a density of 0.16 ganglia/well for survival experiments. For the first 6-7 days in culture 10μM cytosine arabinoside was supplied to eliminate non-neuronal cells. Cultures were maintained in NGF for 7 days prior to the experiments.

2.3 *NGF withdrawal experiments* - Neurons cultured in 96- or 24 well plates for 7 days were deprived of NGF by supplementing medium containing no NGF but with 24 nM anti-NGF antibody (α-NGF) to eliminate trace amounts of NGF that may have been left. C_6-ceramide and C_6-dihydroceramide were added to the medium in dimethyl sulfoxide. Control cultures received equivalent amounts of vehicle to a final concentration never exceeding 0.3% (v:v). Neurons treated with *threo/erythro* –PPMP were given the inhibitors at day 4 in culture and for 3 consecutive days in medium to allow for enzymatic inhibition and ceramide accumulation (de Chaves et al., 1997). On day 7, PPMP-treated cultures were deprived of NGF as before with the addition of PPMP during the deprivation period. After the appropriate times samples were processed for assessment of apoptosis, phosphatase activity or pRb phosphorylation

2.4 *Detection of apoptosis* - Apoptosis was assessed by nuclear staining with Hoechst 33258 (Sigma). Briefly, neurons were washed in ice-cold PBS, fixed for 20min on ice in 4% paraformaldehyde, and incubated with 500 ng/ml Hoechst for 10-20 min. The percentage of apoptotic neurons was calculated by counting the number of condensed and or fragmented nuclei. Unless specified, the total

number of apoptotic neurons included those with fragmented nuclei and "ghost cells" identified by phase contrast and by the absence of stained chromatin. Alternatively, we evaluated loss of mitochondrial membrane potential using MitoTracker® green and orange. Staining was performed according to manufacture's instructions (Molecular Probes, Eugene OR). MitoTracker® Green is essentially non-fluorescent in aqueous solutions and fluoresces at nanomolar concentrations upon accumulation of dye in the lipid membrane of mitochondria. Labeling of mitochondria with MitoTracker® Green is independent of mitochondrial membrane potential. MitoTracker® Orange is a reduced probe which does not fluoresce until entering an actively respiring cell. Oxidation of MitoTracker® Orange converts the dye to a red fluorescent probe which is sequestered in the mitochondria. Therefore only mitochondria that have not lost mitochondrial membrane potential will accumulate MitoTracker® Orange. Images of green and red fluorescence were taken and merged. Neurons negative for MitoTracker® Orange appear green in the merged images and represented cells that had lost mitochondrial membrane potential. Neurons positive for MitoTracker® Orange appear orange and represented live cells. Neurons were visualized using a Nikon Eclipse TE300 inverted fluorescence microscope equipped with Nikon digital camera DXM-1200 (Nikon Canada, Toronto ON), Images were analyzed using Northern Elite V6.0 image capture and analysis software (Empix Imaging, Missisauga, ON).

2.5 ***Preparation of phosphatidic acid*** – 1 mg of Phosphatidic acid (1,2-Di [9cis-9-octadecenoyl]-sn-glycero-3-phosphate) dissolved in chloroform was dried under a constant low-pressure nitrogen stream for 30 mins. Following drying, phosphatidic acid was rehydrated in 50mM Tris-HCl pH 7.4, for 1h at room temperature prior to probe sonication. Sonication was performed using a Vibra Cell 50 probe sonicator (Sonics & Materials, Newtown CT). Phosphatidic acid was sonicated at 15% power in 4 30sec pulses with 30sec of cooling on ice between each pulse.

2.6 ***Determination of phosphatase activity*** – Phosphatase activity in neuronal cell lysates was determined in vitro according to Holmes, 1991. Cellular material from neurons subjected to diverse treatments in 24-well dishes was harvested on ice in 50mM Tris-HCl, pH 7.4 containing leupeptin (1μg/ml), pepstatin (0.1μg/ml), PMSF (1mM), aprotinin (0.5μg/ml) and centrifuged at 14,000 rpm for 5 min at 4°C. Pellets were washed 3 times in ice-cold phosphate buffer saline and resuspended in 40μl 50 mM Tris-HCl (pH 7.4) and lysed by passage through a 100μl Hamilton syringe 10-12 times. Protein content was determined using BCA Assay protein kit (Pierce, Rockford, IL). Protein phosphatase activity was assayed using 10μM [^{32}P] phosphorylase *a* substrate and measuring release of soluble phosphate as described previously (Holmes, 1991). The assay was standardized to obtain a maximum of 15% release of total phosphate from the substrate under control conditions. Values for release of phosphate were

converted to percent of control activity. Assays were performed in duplicate for each sample and duplicate samples were used.

2.7. ***SDS-PAGE and Immunobloting.*** The phosphorylation state of retinoblastoma protein was examined by immunoblot analysis. Neurons cultured in 24 well-dishes and deprived of NGF and treated with or without C_6-ceramide or PPMP were washed in ice-cold PBS containing leupeptin (1μg/ml), pepstatin (0.1μg/ml), PMSF (1mM), aprotinin (0.5μg/ml), and sodium orthovanadate (1mM), and mechanically harvested in a modified RIPA buffer (25mM Tris-HCl, pH 7.4, 50mM NaCl, 0.5% sodium deoxycholate, 2% NP-40, 0.2% SDS, 1mM PMSF, 50mM NaF, leupeptin (1μg/ml), pepstatin (0.1μg/ml), PMSF (1mM), aprotinin (0.5μg/ml), and sodium orthovanadate (1mM). Cell lysates were mixed with 5 times sample buffer to give a final concentration of 2 times sample buffer. 50μl of cell lysate was loaded into each lane and proteins were separated on 7.5% SDS-PAGE. Gels were transferred overnight to PVDF membranes and blocked in tris buffered saline containing 0.1% Tween-20 and 5% non-fat skim milk. Identification of pRb was performed by using polyclonal anti-Rb c-15 antibody (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA) followed by polyclonal rabbit anti IgG (1:5000 Pierce, Rockford IL). Bands were visualized using Super-Signal West Femto Substrate (Pierce, Rockford IL). Molt-4 cell lysates (Santa Cruz Biotechnology) were used as controls. Tubulin was used to control for equal loading by probing with monoclonal anti-α/β tubulin antibody (1:1000) (Sigma).

2.8 *Statistical Analysis* – Statistical analysis of data was performed using Graph Pad Prism Version 3.03 (GraphPad Software Inc., San Diego, CA). Plotted histograms are representative of three or more independent experiments that produced the same results. Unless specified otherwise in individual figure legends, data were plotted as the mean of measured value ± standard deviation of the mean. Significance level (p values) calculated from application of the Student's t-test. For western blot data, the immunoblot presented in this report as "figures" are representative immunoblots of three experiments displaying similar results.

3) RESULTS

3.1) Ceramide inhibits apoptosis of NGF-deprived sympathetic neurons

In the absence of NGF, cultured sympathetic neurons undergo apoptosis with nearly complete population death observed in 48h (reviewed in (Deshmukh and Johnson, 1997). Previous work has shown that short chain ceramide (C_2- or C_6- ceramide) is able to block the neuronal death program initiated by NGF-deprivation (Ito and Horigome, 1995; de Chaves et al., 1997; Nair et al., 2000). However, early studies have not looked at specific hallmarks of apoptosis and

the anti-apoptotic effect of ceramide in sympathetic neurons had not been characterized. We therefore examined if the anti-apoptotic action of ceramide in NGF-deprived sympathetic neurons was dependent on ceramide concentration. 7d old sympathetic neurons cultured in 96 well dishes were deprived of NGF and treated with increasing concentrations of C_6-ceramide or the structurally related isomer C_6-dihydroceramide (C_6-DHCer) for 36h (Fig.4A). At that time, neurons were fixed and stained with the fluorescent nuclear dye Hoechst 33258 to evaluate nuclear fragmentation, a hallmark of apoptosis. Neurons maintained in NGF exhibited intact, round nuclei that were uniformly labeled by the fluorescent probe (Fig.4A). After NGF-deprivation, nuclei appeared condensed and fragmented, and the fluorescence of these nuclei appeared brighter (Fig. 4A). However, when neurons were deprived of NGF and treated together with 20μM C_6-ceramide these neurons exhibited nuclei with similar morphological appearance and fluorescent labeling of those maintained in NGF (Fig. 4A). In contrast, treatment of NGF-deprived neurons with C_6-DHCer did not prevent nuclear fragmentation (Fig. 4A). Ceramide inhibited apoptosis of NGF-deprived sympathetic in neurons in a concentration-dependent manner. In neurons treated with 10μM C_6-ceramide ~80% of the neurons had fragmented nuclei. In contrast, treatment with 20μM C_6-ceramide caused a dramatic reduction in the proportion of neurons with fragmented nuclei (Fig.4B). To confirm the protective effect was specific to ceramide, we tested in parallel the effect of 20μM C_6-DHCer a structural isomer of ceramide lacking the 4-5 trans double bond required for biological activity (Bielawska et al., 1993). The effect was specific to ceramide, as

C_6-DHCer did not inhibit nuclear fragmentation at any concentration tested (Fig. 4B).

In addition, work in our laboratory showed that C_6-ceramide is able to prevent caspase-3 activation, another important hallmark of apoptosis. (Song and Posse de Chaves, 2002). We analyzed whether ceramide was able to block another critical event that occurs during apoptosis, which is the loss of mitochondrial membrane potential (Deshmukh et al., 2000). To asses the status of the mitochondrial membrane potential we used a modification of the MitoTracker® Orange staining method, which has recently been validated as an indicator of mitochondrial membrane potential in sympathetic neurons (Deshmukh et al., 2000). Sympathetic neurons cultured for 7 days in 96-well dishes were deprived of NGF and treated with 20μM C_6-ceramide. In addition to short chain ceramide analogs, we tested whether the natural long chain ceramides generated by the *de novo* pathway were able to support survival. For that purpose, we utilized *threo*-PPMP (*t*-PPMP), a potent inhibitor of glucosylceramide synthase (Fig.2), which causes accumulation of *de novo* synthesized ceramide by blocking conversion of ceramide into glucosylceramide (de Chaves et al., 1997). The *erythro* isomer of PPMP (*e*-PPMP), which does not inhibit glucosylceramide synthase, was used as a negative control. Therefore, some cultures were treated with 5μM *t*-PPMP or 5μM *e*-PPMP for 3 days prior to NGF deprivation to allow accumulation of ceramide. The inhibitors were also included in the corresponding cultures during the deprivation period. 36h after NGF withdrawal neurons were evaluated for the loss of mitochondrial membrane

potential using the fluorescent mitochondrial probe MitoTracker®. MitoTracker® Green is a dye that becomes fluorescent upon accumulation in mitochondrial membranes and labels mitochondria independently of mitochondrial status and therefore will stain total mitochondria (green fluorescence). MitoTracker® Orange is a non-fluorescent dye until oxidized by respiring cells, labeling only intact mitochondria in which membrane potential has not been compromised (red fluorescence). By merging both fluorescence images two populations of neurons can be identified, green neurons that had lost mitochondrial potential and orange neurons bearing intact mitochondria. Most mitochondria in neurons maintained in NGF had normal mitochondrial membrane potential and appeared orange (Fig. 5). Conversely, neurons deprived of NGF showed loss of mitochondrial membrane potential and as a consequence they were green (Fig. 5). Treatment of NGF-deprived neurons with C_6-ceramide or *t*-PPMP caused a dramatic decrease in the number of neurons that lost mitochondrial membrane potential confirmed by an increase in the number of orange neurons when compared with neurons deprived of NGF (Fig.5). The number of neurons that lost mitochondrial membrane potential upon NGF deprivation was equivalent to the number of neurons that contained fragmented nuclei for each of the treatments considered (Fig. 6). C_6-DHCer and *e*-PPMP were unable to prevent the loss of mitochondrial membrane potential that accompanied NGF deprivation (Fig. 6). This experiment suggests that elevation of ceramide levels by means of treatment with short chain analogs or accumulation of natural long chain ceramides prevent mitochondrial changes that occur late in the apoptotic pathway. Taken together

with additional evidence from our lab (Song and Posse de Chaves, 2002) our data indicates that ceramide is a true anti-apoptotic agent in NGF-deprived sympathetic neurons.

3.2) Mechanism of inhibition of apoptosis by ceramide

We were interested in understanding the mechanism by which ceramide inhibits apoptosis. Previous work identified ROS and c-jun as targets for ceramide action (Nair et al., 2000). Events involving ROS and c-jun expression occur early (6-8h) after NGF-deprivation. Consequently, if the protective effect of ceramide is due exclusively to inhibition of c-jun expression, ceramide would not have anti-apoptotic effects when added after 8h of NGF-deprivation. Data from our lab however, showed that 50% of the neurons were protected when ceramide was added 24h after NGF-deprivation (Song and Posse de Chaves, 2002). This value was identical to the value found for NGF re-addition and suggested that ceramide acts by multiple mechanisms, some at early stages of apoptosis, other at later stages.

One possible mechanism of the anti-apoptotic effect of ceramide in sympathetic neurons could be activation of protein phosphatases. We postulated that activation of protein phosphatases and their downstream substrates may be important events in the anti-apoptotic effect of ceramide. Increased phosphatase activity could lead to dephosphorylation of pRb preventing the attempt of cell cycle re-entry that occurs after NGF-deprivation (Farinelli and Greene, 1996). To

determine the effect of ceramide on protein phosphates activity, we measured phosphatase activity in cells treated with or without ceramide or *threo*-PPMP by measuring $^{32}P_i$ released from the phosphatase substrate phosphorylase-a, utilizing the phosphorylase-a phosphatase assay. Sympathetic neurons were treated with 30μM C_6-ceramide or 30μM C_6-DHCer. After 12h of treatment, cellular extracts were prepared and phosphatase activity was measured. Treatment with C_6-ceramide caused an increase in phosphatase activity of approximately 2.5 fold (Fig 7). Conversely, C_6-DHCer did not affect phosphatase activity (Fig 7). In addition to short chain ceramide, the effect of long chain ceramides on protein phosphatase activity was evaluated using PPMP. Sympathetic neurons were treated for three days with 5μM *t*-PPMP or 5μM *e*-PPMP, followed by an additional 12h prior to determination of phosphatase activity. *Threo*-PPMP, but not *erythro*-PPMP elevated protein phosphatase activity (Fig 7). *Threo*-PPMP caused increase of phosphatase activity of approximately 1.9 fold (Fig.7). The values obtained for phosphatase activation by ceramide in our system are similar to those previously reported (Chalfant et al., 1999). Activation of protein phosphatase by endogenous ceramide was as effective as with C_6-ceramide ($p= 0.09$ for C_6-ceramide vs t-PPMP).

To determine the relative contribution of PP-1 and PP2A to the increased phosphatase activity in ceramide treated neurons, we used selective inhibitors of protein phosphatases. Okadaic acid (OA) a marine sponge toxin inhibits PP2A at low concentrations (IC50=0.2nM), and PP-1 at higher concentrations (IC50=20nM) (Bialojan and Takai, 1988), (Dawson and Holmes, 1999). We

tested the effect of 10nM OA added *in vivo*, during ceramide treatment. Neurons were treated with 30μM C_6-ceramide or 5μM *t*-PPMP together with 10nM OA *in vivo*. After 12h, cell extracts were prepared and phosphatase activity was measured as before. OA did not prevent phosphatase activation in neurons treated with C_6-ceramide or *t*-PPMP (Fig 8A). We also tested phosphatidic acid (PA) a selective PP-1 inhibitor (Chalfant et al., 1999), (Kishikawa et al., 1999), (Jones and Hannun, 2002) by adding 30 μM PA together with ceramide during the 12 h incubation period. PA abolished phosphatase activation caused by C_6-ceramide (Fig 8A). However, PA did not inhibit the activation of protein phosphatases by endogenous ceramides (Fig 8A). This experiment suggests that ceramide is likely activating PP-1. Interestingly, in neurons maintained with NGF, OA did not inhibit phosphatase activity and PA inhibited more than 50% of basal phosphatase activity suggesting that most of the phosphatase activity measured with this assay in sympathetic neurons under our culture conditions represents PP-1.

In addition to testing the phosphatase inhibitors *in vivo*, inhibitors were also tested *in vitro*. Cell extracts were prepared from neurons treated with or without C_6-ceramide. The effectiveness of phosphatase inhibitors was tested by adding OA or I-2 for 30 min previous to, and during the enzymatic assay. The addition of 2nM OA to extracts of ceramide-treated neurons caused a statistically non-significant decrease in phosphatase activity (Fig.8B). This is in agreement with the data obtained *in* vivo. On the contrary, the addition of 200nM I-2 during the enzymatic assay dramatically blocked phosphatase activity in ceramide

treated neurons. The combination of 2nM OA and 200nM I-2 produced an almost complete inhibition of phosphatase activity (Fig.8B). Interestingly, OA *in vitro* was unable to decrease phosphatase activity in NGF controls supporting the notion that the majority of the phosphatase activity measured corresponded to PP-1. These experiments suggest that in sympathetic neurons, both short chain ceramide and endogenous ceramide activate protein phosphatases and that the majority of the phosphatase activity in sympathetic neurons is PP-1 or a PP-1 like phosphatase.

3.3) Inhibition of PP-1 blocks the anti-apoptotic effect of C_6-ceramide in sympathetic neurons

The results described above recognize PP-1 as a potential target for ceramide in sympathetic neurons and propose that activation of this target might be relevant in the inhibition of apoptosis. Since we identified PA as an inhibitor of ceramide-activated protein phosphatase in sympathetic neurons, we expected PA to block the anti-apoptotic action of C_6-ceramide if PP1 is involved in the mechanism of action of ceramide. We therefore examined the effect of PA on the anti-apoptotic action of ceramide. Sympathetic neurons cultured for 7 days in 96-well dishes were deprived of NGF and treated with 20μM C_6-ceramide or 5μM

threo-PPMP in the presence or absence of 30μM PA. (Neurons that received PPMP were pretreated for 3 days with the inhibitor). After 36h, cultures were fixed and stained with MitoTracker (as in Fig.5). PA dramatically reduced the anti-apoptotic effect of C_6-ceramide (Fig.9). The percentage of post merge green neurons treated with C_6-ceramide together with PA was similar to NGF-deprived controls, suggesting PA may completely block the anti-apoptotic effect of C_6-ceramide. Cell death in neurons given PA together with *t*-PPMP was more pronounced than in those given *t*-PPMP alone, however when compared statistically they were significantly different than neurons receiving α-NGF alone, indicating that PA was not able to completely abolish the anti-apoptotic effect of *t*-PPMP. Treatment of NGF-controls with 30μM PA did not lead to an increase in apoptotic neurons, nor did treatment with 30μM PA alter the number of apoptotic neurons in NGF-deprived controls (Fig.9). These results suggest that activation of PP-1 by C_6-ceramide is a crucial event in the protective effect of ceramide in NGF-deprived sympathetic neurons.

3.4) pRB undergoes hyperphosphorylation during NGF-deprivation

The "cell cycle" hypothesis establishes that upon NGF-deprivation sympathetic neurons attempt to re-enter the cell cycle. Consequences of this attempted re-entry, in other neurons are activation of CDKs and hyperphosphorylation of pRb (Appendix A). Although the protective effect of inhibitors of the cell cycle and CDK inhibitors has been established (Farinelli and

Greene, 1996), (Park et al., 1996) hyperphosphorylation of pRb has not been directly demonstrated in sympathetic neurons deprived of NGF.

We therefore examined the phosphorylation status of pRb in sympathetic neurons during NGF-deprivation. Sympathetic neurons cultured for 7 days were deprived of NGF for times varying from 6-18h. At the indicated times neurons were processed for SDS-PAGE and immunoblot analysis. To determine pRb phosphorylation status we utilized an antibody directed against the spacer region of the pRb protein, allowing detection of multiple pRb species independent of phosphorylation state. To confirm identity of detected bands, a pRb overexpressing Molt-4 lysate was used as a positive control (not shown). Electrophoresis of lysates from sympathetic neurons deprived of NGF resolved multiple bands corresponding to hyper- and hypo-phosphorylated pRb (Fig.10). A hyperphosphorylated form of pRb appeared 12h after NGF-deprivation and was more prominent at 18h of deprivation. Hyperphosphorylated pRb bands were not detected in lysates of sympathetic neurons which had not been deprived of NGF, confirming that hyperphosphorylation of pRb is a consequence of NGF deprivation. The differences observed cannot be attributed to differences in total protein content since immunoblotting for tubulin indicated similar amounts of protein for all treatments. These experiments suggest that one consequence of depriving sympathetic neurons of NGF is pRb hyperphosphorylation.

3.5) Ceramide prevents pRb hyperphosphorylation caused by NGF-deprivation

Immunoblot analysis of sympathetic neurons-cell lysates revealed a time-dependent hyperphosphorylation of pRb. pRb is a natural substrate for PP-1 and we showed that PP-1 activity is increased in sympathetic neurons treated with C_6-ceramide. Thus, we examined whether pRb was a substrate of ceramide-activated PP-1. Sympathetic neurons cultured for 7 days were deprived of NGF and treated with 30μM C_6-ceramide for 6-18h. At indicated times cellular material was harvested and processed for immunoblot analysis. Contrary to NGF-deprived sympathetic neurons, hyperphosphorylated pRb was undetectable in sympathetic neurons treated with 30μM C_6-ceramide in the absence of NGF (Fig.11A). Only a band corresponding to hypophosphorylated pRb was present (Fig. 11A). The absence of hyperphosphorylated bands of pRb was not a result of changes in the amount of total protein as demonstrated by tubulin immunobloting detection. The results presented above indicate that treatment of NGF-deprived sympathetic neurons with ceramide inhibits the hyperphosphorylation of pRb.

We next examined if inhibition of PP-1 could block the effect of ceramide on pRb dephosphorylation. 7 day-old neurons were deprived of NGF and treated with 30μM C_6-ceramide or 30μM C_6-ceramide together with 30μM PA for 6-18h (Fig. 11B). At the indicated times the presence of hyperphosphorylated forms of pRb was analyzed as before. C_6- Ceramide was not able to prevent pRb

hyperphosphorylation in NGF-deprived neurons when given together with PA (Fig. 11B). Interestingly, hyperphosphorylation of pRb in neurons treated with ceramide and PA occurred as early as 6h.

In addition to C_6-ceramide, the effect of endogenous ceramide on pRb phosphorylation was investigated. 4 day-old neurons were treated with 5μM *threo*-PPMP for 3d in to induce endogenous ceramide accumulation. On day 7, neurons were deprived of NGF and 5μM *threo*-PPMP was supplied for 6-18h. At the indicated times neurons were processed for immunobloting of pRb. Similar to C_6-ceramide, *threo*-PPMP prevented the hyperphosphorylation of pRb that occurs after NGF deprivation (Fig. 12A). However, when we tested the ability of PA to block the effect of *threo*-PPMP, we found that PA did not prevent the effect of *threo*-PPMP to inhibit pRb hyperphosphorylation (Fig. 12B). These experiments suggest that pRb is dephosphorylated in response to endogenous ceramide, but that this effect is not inhibited by treatment with PA.

4) DISCUSSION AND CONCLUSION

The objective of this project was to evaluate the role of serine/threonine-directed protein phosphatases as targets of ceramide for blocking apoptosis in sympathetic neurons. We hypothesized that ceramide activates PP-1 and that such activation leads to pRb dephosphorylation in the absence of NGF. To

examine this hypothesis we used the classic model of apoptosis in cultured sympathetic neurons deprived of NGF.

Ceramide inhibits apoptosis in NGF-deprived sympathetic neurons

In neurons ceramide is either neuroprotective (Goodman and Mattson, 1996), or neurotoxic (Brugg et al., 1996; Ping and Barrett, 1998). In sympathetic neurons, ceramide has previously been shown to inhibit apoptosis (Ito and Horigome, 1995; de Chaves et al., 1997; Nair et al., 2000; de Chaves et al., 2001). However, the mechanism by which ceramide protects NGF-deprived sympathetic neurons is not fully understood. Nair *et al.* recently reported that short chain ceramide analogs block oxidative stress and induction of the c-jun transcription factor in sympathetic neurons undergoing apoptosis in the absence of NGF (Nair et al., 2000). This represents one potential anti-apoptotic mechanism by which ceramide blocks early events associated with NGF-deprivation (Deshmukh and Johnson, 1997; Chang and Johnson, 2002). However, work from our laboratory suggests that ceramide may act both at early and late phases of the apoptotic program. Song and Posse de Chaves showed that sympathetic neurons deprived of NGF were protected from apoptosis if ceramide treatment immediately followed NGF deprivation and that ceramide was also effective in blocking apoptosis when supplied up to 24h following NGF-withdrawal (Song and Posse de Chaves, 2002). The finding that ceramide still protects sympathetic neurons from apoptosis at a much later time than when

induction of oxidative stress and c-jun activation occurs 16-18h after NGF-deprivation, prompted us to further characterize the anti-apoptotic effect of ceramide and explore potential anti-apoptotic mechanisms in addition to those proposed by Nair et al.

Therefore, we examined the effect of ceramide on multiple events associated with apoptosis. Previous work in our laboratory showed that ceramide inhibits nuclear fragmentation and activation of caspase-3, hallmarks of apoptosis (Song and Posse de Chaves, 2002). Here we investigated whether ceramide could block the loss of mitochondrial membrane potential that occurs during NGF-deprivation (Deshmukh et al., 2000). We found that both short chain ceramide and endogenous ceramide inhibited loss of mitochondrial membrane potential in the absence of NGF. In addition, we showed that NGF-deprived neurons, which were protected against the loss of mitochondrial membrane potential, were also protected from nuclear fragmentation. These findings taken together demonstrate that ceramide blocks at least two processes that occur in the final stages of apoptosis in sympathetic neurons.

Therefore, we have further established ceramide as a true anti-apoptotic molecule in sympathetic neurons deprived of neurotrophic support. Our data, however, does not allow us to differentiate whether the loss of mitochondrial potential is due to a direct effect of ceramide at the level of the mitochondria or if ceramide blocks apoptotic signals upstream of loss of membrane potential and subsequent release of cytochrome c. Ceramide has been shown to activate a mitochondria-associated PP2A like phosphatase and dephosphorylate Bcl-2 in

HL-60 cells (Ruvolo et al., 1999). While this finding by Ruvolo *et al* suggests the effect of ceramide at the level of the mitochondria to be pro-apoptotic, it highlights the fact that at least one direct target of ceramide co-localizes with mitochondria. Our data, however suggests that PP2A is not activated in response to ceramide in sympathetic neurons (see below). Interestingly, it has recently been demonstrated that members of the Bcl-2 family proteins are regulatory subunits for PP-1. Ayllon et al showed that Bcl-2, and Bcl-xL can target PP-1 to the mitochondria (Ayllon et al., 2001; Ayllon et al., 2002), and that a consequence of mitochondrial PP-1 activity resulted in Bad dephosphorylation (Ayllon et al., 2000). We have shown that ceramide activates PP-1 in sympathetic neurons (see below). Therefore, one consequence of ceramide treatment may be activation of PP-1 localized on the mitochondrial membrane, which may be involved in preventing loss of mitochondrial potential. In any case, the effect of ceramide in inhibiting loss of mitochondrial membrane potential should be further investigated to define possible mechanisms involved in the anti-apoptotic signaling by ceramide.

Activation of protein phosphatase-1 by ceramide

The other major aim of this project was to explore potential mechanisms that may be involved in the anti-apoptotic effect of ceramide in sympathetic neurons. We examined the role of serine/threonine protein phosphatases as

possible transducers of the anti-apoptotic signal of ceramide. To date relatively few direct targets of ceramide have been identified. The short list includes a ceramide activated protein kinase (CAPK), which has been identified as the kinase suppressor of ras (KSR) (Zhang et al., 1997), cathepsin D (Heinrich et al., 2000), atypical protein kinase C ζ (PKCζ) (Lozano et al., 1994), and ceramide activated protein phosphatases (CAPPs) (Dobrowsky and Hannun, 1992; Chalfant et al., 1999). Initial studies in search of a direct target of ceramide revealed activation of a protein phosphatase by short chain ceramides (Dobrowsky and Hannun, 1992), which shared many biochemical properties with PP2A (Dobrowsky and Hannun, 1993; Chalfant et al., 2000). In addition to PP2A, short and long chain ceramides activate PP-1 (Chalfant et al., 1999; Kishikawa et al., 1999).

We examined the role of CAPPs as possible effectors of ceramide anti-apoptotic mechanism(s) in NGF-deprived sympathetic neurons. Our data indicates that short chain C_6-ceramide and elevation of endogenous ceramide (through use of the glucosylceramide synthase inhibitor *threo*-PPMP) increased by nearly two fold total phosphatase activity in sympathetic neurons. In comparison, the biologically inactive isomers C_6- dihydroceramide and *erythro*-PPMP failed to significantly increase phosphatase activity. The finding that only D-*erythro*-ceramide is a strong activator of protein phosphatases in sympathetic neurons is in agreement with studies demonstrating that the activation of phosphatases by ceramide *in vitro* is highly stereospecific (Dobrowsky et al., 1993; Kishikawa et al., 1999) and requires the presence of the trans 4,5 double

bond. The degree of activation that we found in sympathetic neurons corresponds closely to values published for ceramide-activation of catalytic sub-units of these phosphatases *in vitro* (Dobrowsky et al., 1993; Kishikawa et al., 1999). Ceramide has been shown to activate both PP-1 (Chalfant et al., 1999) and PP2A (Dobrowsky and Hannun, 1993). *In vitro*, short and long chain ceramides activate the catalytic subunit (PP2AC), as well as dimeric (AC) and trimeric (ABαC) complexes of PP2A, and Cα and Cγ catalytic subunits of PP-1. To identify which phosphatases had increased activities in sympathetic neurons in response to ceramide, the differential inhibitor sensitivities of ser/thr phosphatases was exploited. Okadaic acid, a toxin produced from the marine sponge *H.Okadai* is an extremely potent inhibitor of PP2A at concentrations of 0.2-.2.0nM (Bialojan and Takai, 1988), (Dawson and Holmes, 1999). In addition, OA will also inhibit PP-1 at higher concentrations (20-100nM) (Bialojan and Takai, 1988; Dawson and Holmes, 1999). In sympathetic neurons, the lack of sensitivity of ceramide-activated phosphatase activity to 10 nM OA *in vivo* suggested that the majority of phosphatase activity increased by ceramide is not PP2A or a PP2A like phosphatase. OA also failed to inhibit basal phosphatase activity, measured from lysates of sympathetic neurons maintained in NGF, suggesting that the majority of phosphatase activity measured under our experimental conditions in sympathetic neurons does not correspond to PP2A. Furthermore, 2nM OA added to extracts of neurons *in vitro*, immediately prior to determining phosphatase activity also failed to block ceramide-activated phosphatases by C_6-ceramide or basal phosphatase activity in sympathetic

neurons as well. At concentrations greater than 10nM OA became toxic to sympathetic neurons, and therefore OA could not be used to block PP-1. The complete lack of inhibition by OA could suggest inactivity of the compound, however most of the phosphatase activity was blocked using PP-1 inhibitors (see below). Thus, the experiments with OA suggest that most of the activity measured belongs to a class of phosphatases different than PP2A.

To examine the involvement of PP-1 as a ceramide activated phosphatase in sympathetic neurons, phosphatidic acid (PA) an inhibitor of PP-1 was used. Phosphatidic acid has been reported to bind directly to the catalytic subunit of PP-1Cα (Kishikawa et al., 1999), and PP-1Cγ in vitro (Jones and Hannun, 2002). PA inhibits PP-1, but does not inhibit, and may activate PP2A *in vitro* (Kishikawa et al., 1999). PA also reduces the activating effect of ceramide on PP-1 *in vitro* (Chalfant et al., 1999; Kishikawa et al., 1999). Accordingly, we found that *in vivo* PA dramatically blocked the increased activity of phosphatases in sympathetic neurons treated with short chain ceramide. PA also lowered basal phosphatase activity in neurons cultured without ceramide, suggesting that the majority of phosphatase activity we measured in sympathetic neurons is PP-1 or a PP-1 like phosphatase. In addition to PA, we tested the effect of I-2 on phosphatase activity. I-2 is a specific inhibitor of PP-1, with no inhibitory activity towards PP2A. I-2 was tested *in vitro* against neurons treated with C_6- ceramide. I-2 dramatically reduced ceramide activated phosphatase activity and basal phosphatase activity in sympathetic neurons. These results match those of PP-1 inhibitors tested *in vivo* (PA), further suggesting that most of the basal phosphatase activity in

sympathetic neurons is likely PP-1 or a PP-1 related phosphatase and that the activation elicited by ceramide also corresponds to PP1.

When PA was tested *in vivo* against neurons in which natural ceramides was elevated; it had very little effect on reducing phosphatase activity. The ability of PA to inhibit phosphatase activity increased by short chain ceramide but not natural ceramides was unexpected. A few possibilities exist when considering the differential effect of PA on inhibiting phosphatase activity induced by ceramide.

The difference might be explained by the formation of two different pools of ceramide in neurons treated with C_6-ceramide and PPMP. These two different pools could have different cellular locations and PA might have different access to them. Treatment of cells with cell permeable, short chain ceramide analogs will lead to incorporation of the analogs in the plasma membrane, followed by endocytosis into endosomes and eventually accumulation in the Golgi (Babia et al., 2001) with further delivery to the endoplasmic reticulum (Song and Posse de Chaves, 2002). In contrast natural ceramide elevated by use of PPMP, will likely accumulate in the endoplasmic reticulum (Mandon et al., 1992) and possibly the cytoplasmic leaflet of the Golgi. We have not examined the cellular localization of PA after cellular uptake but it seems reasonable that it will get in contact much easier with the pool of short chain ceramide. Moreover, each pool of ceramide may interact with distinct isoforms of PP-1. The expression and localization of the different isoforms of the catalytic subunit of PP-1 has not been established in sympathetic neurons. To fully determine the isoforms of PP-1 relevant in

ceramide activation in sympathetic neurons further studies need to be carried out.

A second possibility relates to the differential effect of PA on PP-1 activated by ceramide of different acyl-chain lengths. *In vitro* PA dramatically blocks the activation of PP-1Cα by C_6-ceramide (Kishikawa et al., 1999), however it sensitizes PP-1 activated by C_{18}-ceramide, decreasing the concentration of long chain ceramides needed to activate the enzyme *in vitro* (Chalfant et al., 1999). Pools of ceramide generated in response to PPMP will contain exclusively long chain ceramides. On the other hand in neurons treated with C_6-ceramide we expect the bioactive pool of ceramide to contain both short and long chain ceramides.

In turn, PA itself is a bioactive molecule. PA is generated from phosphatidylcholine by action of phospholipase D, by acylation of lysophosphatidic acid by lysophosphatidic acid acyltransferase, or by phosphorylation of diacylglycerol by diacylglycerol kinase. While no direct targets for PA have been identified, PA has been suggested to have a role of docking Raf-1 at the plasma membrane, and may interact with phospholipase Cγ, as well as a PA-dependent kinase (Waite et al., 1997; McPhail et al., 1999). The finding that PA activates kinases while also inhibiting phosphatases needs to be considered when utilizing PA as an experimental tool.

Relevance of PP-1 activation in the anti-apoptotic action of ceramide -Role of pRb as a substrate for ceramide activated PP-1

Finally, we examined the relevance of PP-1 activation by ceramide for the anti-apoptotic effect of ceramide in NGF-deprived neurons. Using PA to inhibit PP-1, we found that the anti-apoptotic effect of C_6-ceramide in NGF-deprived neurons dramatically depended on the ability to activate PP-1. Inhibition of PP-1 produced almost a complete blockade of the anti-apoptotic effect of C_6-ceramide. In contrast, but in agreement with our results on PP-1 activation, the anti apoptotic effect of *threo*-PPMP in NGF-deprived neurons was much less affected by PA, and there was a non-significant reduction in the total number of neurons rescued from apoptosis. This differential effect of PA suggests again that PA might not be able to access the pool of ceramide generated by *t*-PPMP.

We also examined physiological substrates of PP-1 in an attempt to elucidate a pathway by which ceramide-activated PP-1 protects NGF-deprived sympathetic neurons from apoptosis. Among the targets of PP-1, pRb emerged as a good candidate that could explain the inhibition of apoptosis according to the "cell cycle" theory. In mammalian cells, the cell cycle includes multiples checkpoints to ensure proper cell proliferation. pRb is part of one of these crucial checkpoints in the cell cycle, controlling transition from G1 to S phase. Regulation of pRb occurs through phosphorylation. Hyperphosphorylated pRb is required for cell cycle progression (Weinberg, 1995), however in non-cycling cells, such as neurons the presence of hyperphosphorylated pRb could signal an

attempt to enter into the cell cycle. This inappropriate attempt to drive the cells through the cell cycle may, in fact activate apoptotic mechanisms within the cell. Heintz originally proposed that deregulation of cell cycle components in neurons would lead to attempted cell cycle re-entry and apoptosis, in what is now recognized as the "cell cycle hypothesis" (Heintz, 1993). Evidence for an attempt to re-enter the cell cycle includes the increased expression of the cell cycle protein cyclin D1, a binding partner for the pRb-directed cyclin-dependent kinases that occurs upon NGF-deprivation (Freeman et al., 1994). In addition, the finding that inhibitors of the cell cycle and inhibitors of CDKs protect sympathetic neurons from apoptosis induced by NGF-withdrawal (Farinelli and Greene, 1996; Park, et al. 1996) suggested de-regulation of cell cycle components during neuronal death.

In agreement, our experiments demonstrated that NGF withdrawal leads to hyperphosphorylation of pRb. Most of the studies in sympathetic neurons focused on CDKs but to our knowledge, our work is the first to directly examine the phosphorylation status of pRb during NGF-deprivation. We found that in sympathetic neurons maintained with NGF, pRb is in a hypophosphorylated state, which corresponds to growth arrest/terminal differentiation (Weinberg, 1995). When neurons were deprived of NGF we observed an increase in hyperphosphorylation of pRb which was evident at 12h following NGF-deprivation, and even more abundant after 18h of deprivation.

Considering that pRb hyperphosphorylation may be a pro-apoptotic signal in sympathetic neurons we examined whether the phosphorylation of pRb in

sympathetic neurons was altered by treatment with ceramide. Ceramide has been previously shown to cause dephosphorylation of pRb in Molt-4 cells (Dbaibo et al., 1995; Kishikawa et al., 1999). Accordingly, our data indicates that both C_6-ceramide and *threo*-PPMP prevented hyperphosphorylation of pRb in NGF-deprived sympathetic neurons.

We examined the possibility that dephosphorylation of pRb in response to ceramide is due to activation of PP-1 since pRb is a substrate for PP-1 (Ludlow et al., 1993). We found that when PA was included together with C_6-ceramide pRb was not dephosphorylated. Interestingly we observed that PA treatment was not effective in blocking the action of *threo*-PPMP in dephosphorylating pRb. This lack of inhibition by PA is in agreement with the inability of PA to block the activation of protein phosphatases by *threo*-PPMP (see above). The finding that PA does not block activity of *threo*-PPMP towards pRb is another indication that the pool of ceramide generated by *threo*-PPMP may localize to a cellular compartment not accessible to PA. Nevertheless, our experiments suggest that the mechanism by which C_6-ceramide dephosphorylates pRb is through activation of PP-1.

Although we have demonstrated that pRb is hyperphosphorylated upon NGF-deprivation, and that ceramide treatment reverses this effect, we do not have direct evidence that supports pRb dephosphorylation as an anti-apoptotic event.

Alternative targets for ceramide activated protein phosphatase-1

It is possible that in addition to dephosphorylation of pRb in NGF-deprived sympathetic neurons, the increased PP-1 activity in response to ceramide causes dephosphorylation of other multiple substrates of PP-1. Therefore, downstream targets of PP-1 other than pRb may be involved in the anti-apoptotic effect of ceramide in sympathetic neurons. Substrates of PP-1 that are involved in regulation of apoptosis are the SR proteins a family of serine/arginine rich RNA splicing factors (Mermoud et al., 1994), (Misteli and Spector, 1996). SR proteins are involved in spliceosome assembly and alternative mRNA splicing (Misteli and Spector, 1996), (Zahler et al., 1992; Zahler et al., 1993). The function of SR proteins is dependent on phosphorylation state. Endogenous ceramide has been shown to dephosphorylate these proteins in a PP-1 dependent manner (Chalfant et al., 2001). In addition, activation of PP-1 by ceramide has been shown to influence alternative splicing of two regulators of apoptosis, caspase-9 and Bcl-xL (Chalfant et al., 2002). Therefore, it may be relevant to consider other substrates of PP-1 when examining the anti-apoptotic mechanism of ceramide in sympathetic neurons.

Final Remarks

In summary, our findings show that ceramide activates PP-1 in sympathetic neurons and moreover, inhibition of PP-1 blocks the anti-apoptotic

effect of C_6-ceramide in these neurons. We also show that pRb a PP-1 substrate is hyperphosphorylated in response to NGF-deprivation, and that this hyperphosphorylation can be reversed by treatment with ceramide. Taken together these findings suggest PP-1 as an important target for ceramide in sympathetic neurons.

Further examination of the role of ceramide as an anti-apoptotic agent in neurons is warranted as tool to better understand mechanisms of neuronal death. Defects in regulation of apoptosis contribute to a variety of diseases, and unregulated apoptosis has been implicated in neurodegenerative disease as well as stroke and some AIDS associated pathologies. Ceramide protects sympathetic neurons from death in a manner similar to caspase inhibitors (BAF), in which neurons appear atrophic but retain viability and are responsive to the re-addition of NGF. Therefore, protective agents of this nature may be valuable following acute insults to the nervous system, in which with time a normal cellular enviroment is restored (Deshmukh et al., 1996), (Holtzman and Deshmukh, 1997). Several therapeutic drugs currently in use in the market cause elevation of ceramide (reviewed in Senchenkov et al., 2001), and other drugs being examined in pre-clinical trials may prove useful in "sphingolipid therapy" (Kolesnick, 2002). These circumstances, take together with the cell-specific effects of ceramide emphasize the relevance of understanding the molecular mechanisms by which ceramide regulates apoptosis.

Appendix A

Brief overview of cell cycle regulation

The mammalian cell cycle consists of five phases: two gap or growth phases (G_1 and G_2), a phase in which DNA synthesis occurs (**S**), a mitotic phase in which cells divide (**M**), and an arrested phase in which cells are not actively dividing (G_0) Fig. A. The "cyclic" nature of this process is represented by the fact that newly formed cells exiting from mitosis enter into G_1 to continually increase cell number until there are signals to arrest cellular proliferation or undergo terminal differentiation.

Progression though the cell cycle is controlled by successfully traversing two checkpoints. The initial checkpoint occurs at the end of G1 prior to S phase entry (G1-S transition). This checkpoint is regulated by the retinoblastoma protein (pRb) Fig. A. The second checkpoint occurs at the G2-M transition and is regulated by the p53 transcription factor. Relevant to this thesis only pRb's function as a cell cycle regulator will be considered.

pRb and cell cycle control (reviewed in Weinberg, 1995)

pRb is the initial checkpoint in cell cycle progression. The control of cellular proliferation through pRb is regulated by the phosphorylation state of this protein. In a hyperphosphorylated state pRb is *active*, and inhibits proliferation. In the

hypophosphorylated state pRb binds a variety of cellular proteins, most notably being the E2F family of transcription factors. Upon mitogenic signals, pRb is sequentially hyperphosphorylated on multiple serine/threonine residues, *inactivating* pRb, causing release of E2F. Free E2F transcription factors upregulate genes and proteins required for S phase entry. Inhibitory phosphorylation of pRb is catalyzed by family of ser/thr directed cylcin-dependent kinasess (CDKs) (Fig.A and for review on CDK function see Sherr, 1993). CDKs require an activating protein, termed cyclins, and the kinase-cyclin complex is active in phosphorylating pRb. Re-activation of pRb does not occur until the inhibitory phosphates are removed at which point hyperphosphorylated pRb complex with E2F and other cellular proteins to arrest the cell cycle. Removal of the inhibitory phosphate is catalyzed by protein phosphatase-1 (PP-1)

PP-1 and pRb ***(reviewed in detail in Tamrakar et al., 2000)***

Re-activation of pRb to a growth inhibitory state is catalyzed by PP-1 (Fig. A). PP-1 is a ser/thr directed phosphatase, which dephosphorylates hyperphophorylated pRb at specific stages of the cell cycle. CDKs, which phosphorylate pRb to intiate cell cycle progression, also phosphorylate PP-1, inhibiting phosphatase function during cell cycle stages when hyperphosphorylated pRb is required.

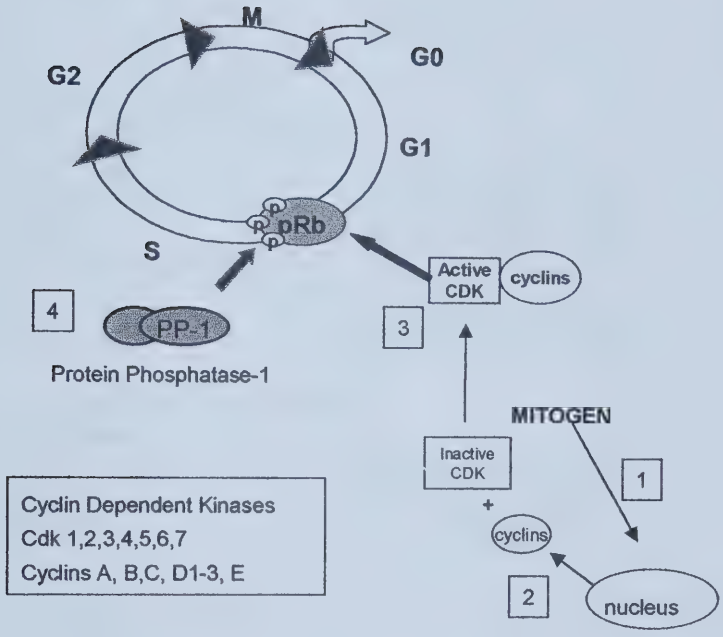

Fig. A Cell cycle regulation 1) Mitogens induce cyclin synthesis. 2) Cyclins bind and activate CDKs 3) Active CDKs phosphorylate pRb allowing cell cycle progression 4) PP-1 dephosphorylates pRb, inhibiting cell cycle progression

Table.1 Regulatory subunits of PP-1

Regulatory Subunit	Function
Specific Substrates	
pRb	Cell cycle progression
53BP2 (p53- binding)	Cell cycle checkpoint
Mitochondrial Targeting	
Bcl-2	Apoptosis (Bad dephosphorylation)
Plasma Membrane/ Cytoskeleton Targeting	
Neurabin I	Neurite outgrowth
Spinophilin (neurabin II)	Synaptic transmission
NF-L	Synaptic transmission
BH-prootocadherin-c	Neuronal cell-cell interaction
Yotiao	Synaptic transmission
Endoplasmic Reticulum Targeting	
L5	Protein synthesis
RIPP1	Protein synthesis
GADD34	Protein synthesis
Spliceosomal/RNA Targeting	
NIPP1	Pre-mRNA splicing
PSF1	Pre-mRNA splicing
p99 (PNUTS)	RNA processing
Hox11	Cell cycle checkpoint
HCF	Transcription, cell cycle
Glycogen Processing	
GM	Glycogen metabolism
GL	Glycogen metabolism
Inhibitory Molecules	
Inhibitor-1	Catalytic Inhibition
Inhibitor-2	Catalytic Inhibition
DARPP-32	Catalytic Inhibition

Adapted from Cohen, P.T. 2002. Protein phosphatase 1--targeted in many directions. *J Cell Sci.* 115:241-56

Table. 2 Regulatory subunits of PP2A.

Regulatory subunit
SV40 small T antigen
Adenovirus E4 antigen
PR55/B
PR56/61/B`
PR48/B``
PR59/B``
PR72/B``
PR130/B``
A/R52

Adapted from Barford, D., A.K. Das, and M.P. Egloff. 1998. The structure and mechanism of protein phosphatases: insights into catalysis and regulation. *Annu Rev Biophys Biomol Struct*. 27:133-64.

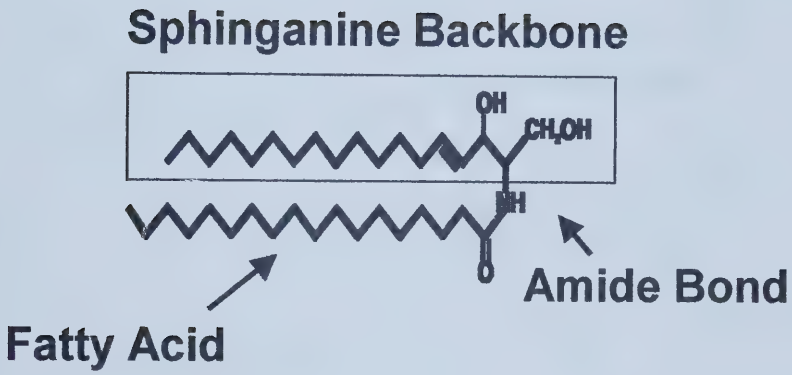

Fig. 1 Structure of ceramide

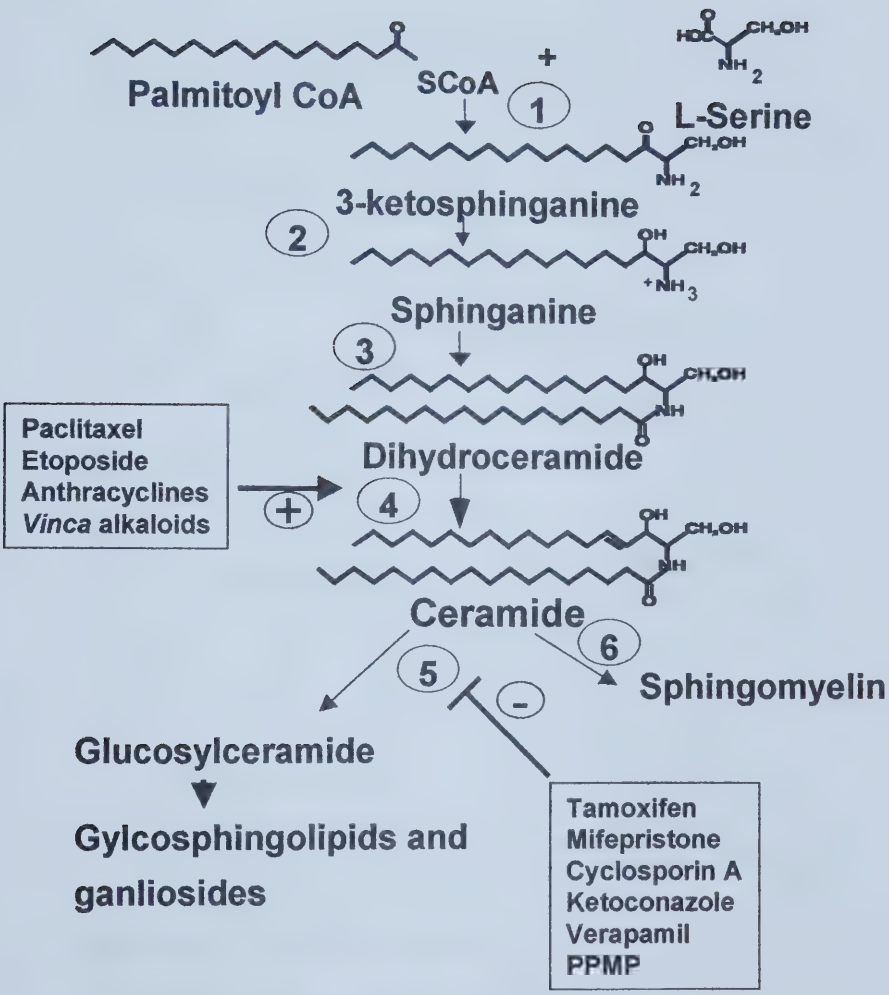

Fig.2 - ***De novo*** **synthesis of ceramide**

Enzymes are as follows

1) Serine palmitoyl transferase 2) 3-ketosphinganine reductase 3) ceramide synthase/ sphinganine N-acyl transferase 4) dihydroceramide desaturase 5) glucosylceramide synthase 6) ceramide cholinephosphotransferase

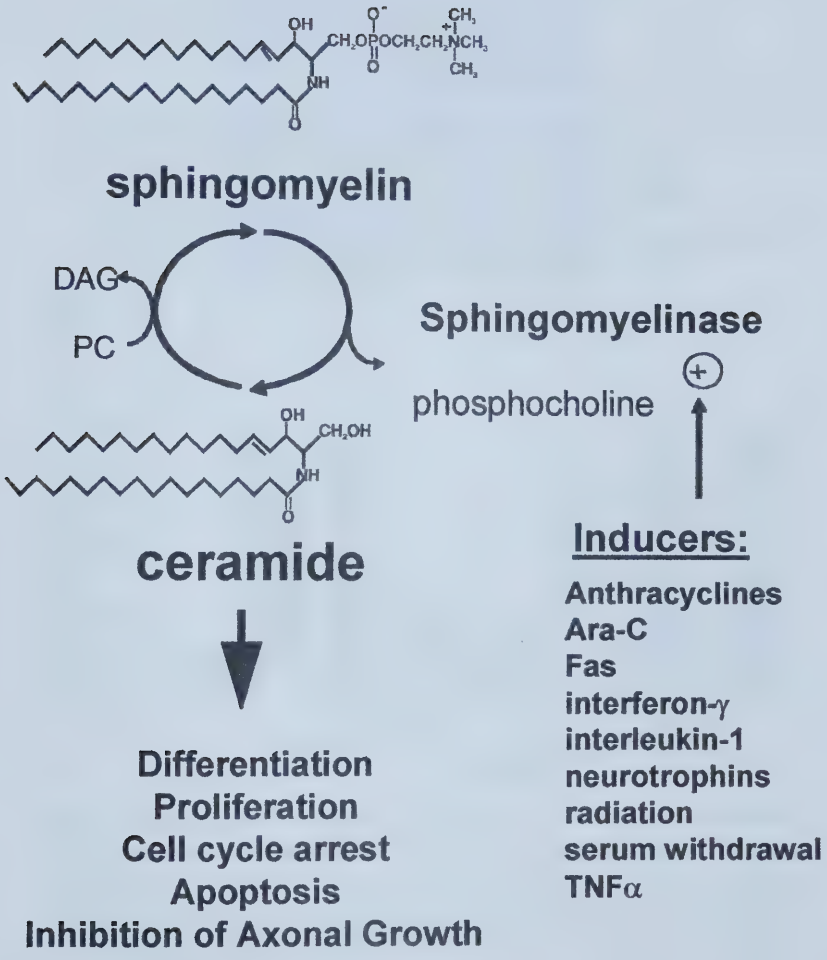

Fig. 3 The sphingomyelin cycle

Enviromental stimuli and agonist-activated receptors activate sphingomyelinases which hydrolyze membrane sphingomyelin generating ceramide and phosphocholine.

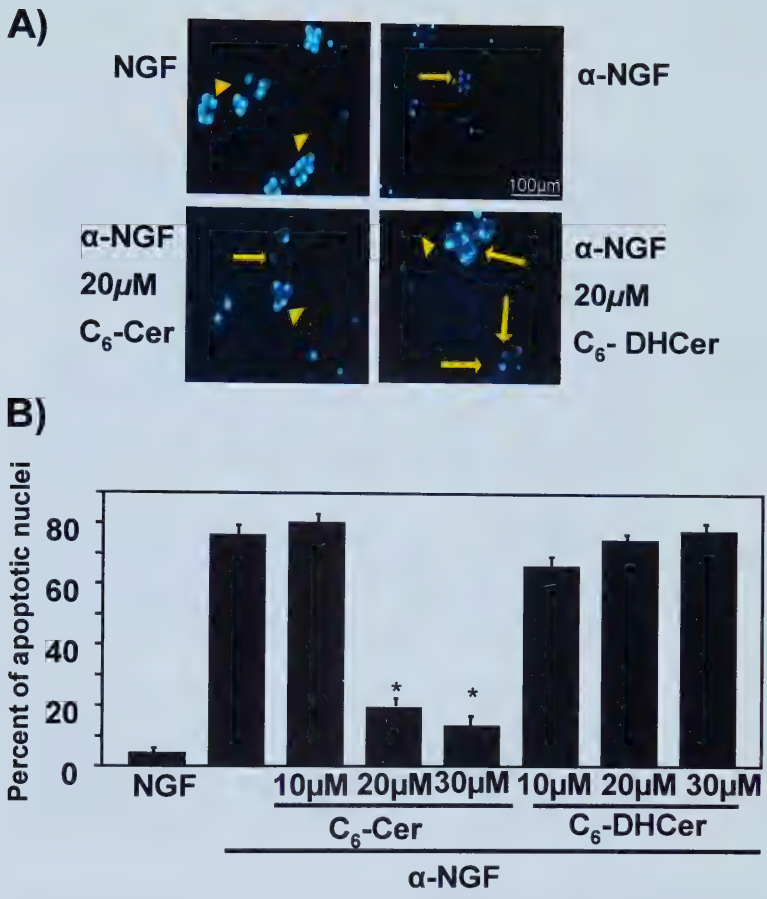

Fig.4 Ceramide inhibits apoptosis in NGF-deprived sympathetic neurons Rat sympathetic neurons were cultured in 96 well dishes for 7d in the presence of 50ng/ml NGF. At that time neurons were deprived of NGF and treated with 24nM NGF neutralizing antibody (α-NGF) or α-NGF and increasing concentrations of C_6-ceramide (C_6-Cer) or C_6-dihydroceramide (C_6-DHCer). After 36h neurons were fixed and stained with the nuclear dye Hoechst 33258. **A)** Representative photomicrographs of relevant treatments. Arrowheads indicate normal nuclei, arrows indicate apoptotic nuclei **B)** Quantitation of A). Results are mean ± S.D. of three experiments. Each experiment consisted of 4 wells/treatment. >500 neurons were counted per treatment * significantly different from cultures treated with α-NGF ($p<0.001$) as calculated by the students t-test.

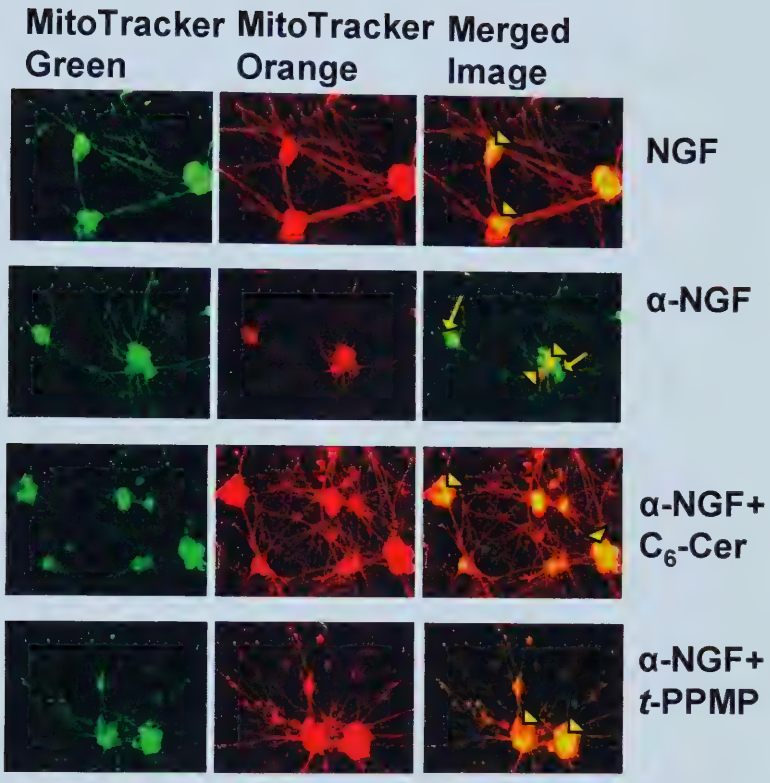

Fig. 5 Ceramide inhibits loss of mitochondrial membrane potential in NGF-deprived sympathetic neurons. Rat sympathetic neurons cultured for 7 days in 96-well dishes in the presence of 50ng/ml NGF were deprived of NGF for 36h and the loss of mitochondrial membrane potential was evaluated with MitoTracker as described in Experimental Procedures. Neurons were cultured for 36h as follows; **NGF**, medium containing 50ng/ml NGF, **α-NGF**, medium deprived of NGF and supplemented with 24nM NGF neutralizing antibody, **α-NGF+C_6-Cer**, NGF free medium containing the anti-NGF antibody and 20μM C_6-ceramide, **α-NGF+*t*-PPMP**, NGF free medium containing the anti-NGF antibody and 5 μM *threo*-PPMP. Neurons receiving *t*-PPMP were pretreated for 3days prior to deprivation. Figure represents fluorescent micrographs. In the merged images arrowheads indicate normal neurons, and arrows indicate apoptotic neurons.

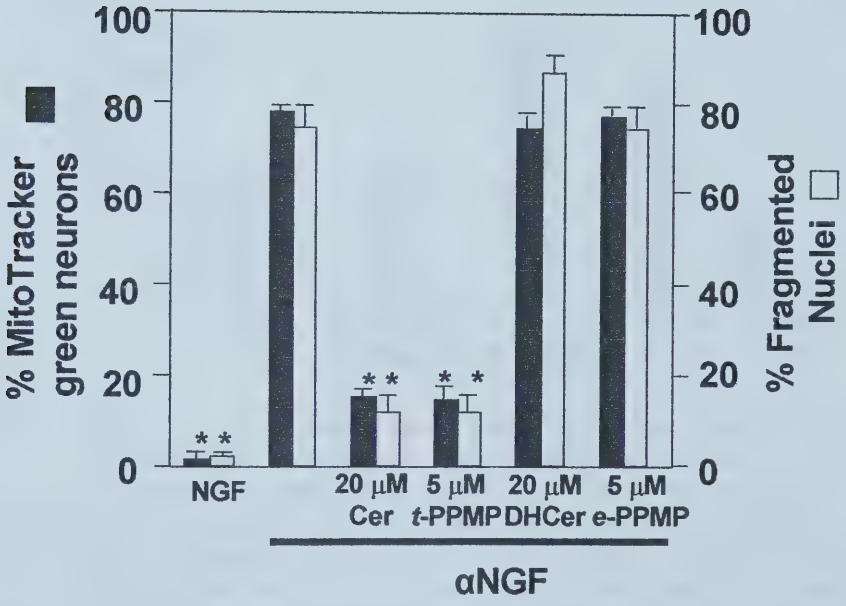

Fig. 6 - Comparison of MitoTracker and Hoechst staining for apoptosis evaluation in sympathetic neurons Neurons were treated as indicated in Fig. 5. In addition some neurons were treated with medium containing αNGF and 30μM C_6-dihydroceramide or 5μM *erythro*-PPMP as negative controls for ceramide and *threo*-PPMP respectively. Nuclear fragmentation was evaluated by Hoechst 33258 staining and compared with MitoTracker Staining. Results are mean ± S.D. of three experiments. Each experiment consisted of 4 wells per treatment. > 500 neurons per treatment were counted. * significantly different as compared to αNGF ($p<0.001$) as calculated by the students t-test.

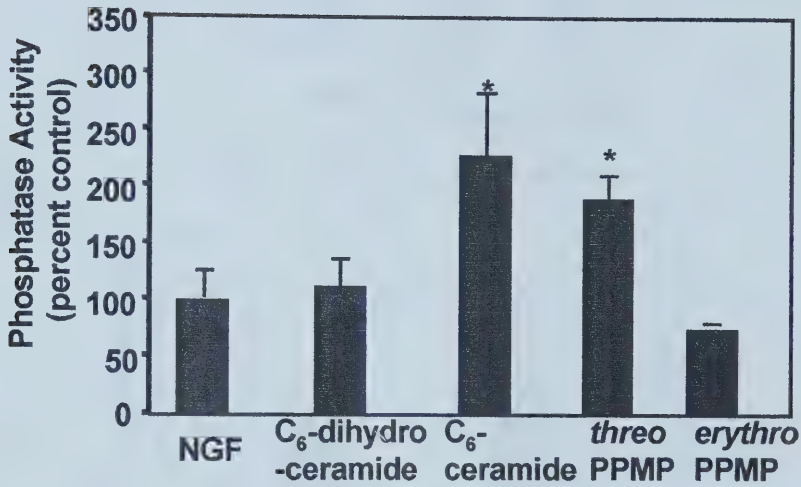

Fig. 7 - C_6-ceramide and *threo*-PPMP increase protein phosphatase activity in sympathetic neurons. 7d old sympathetic neurons were treated with 30μM C_6-ceramide, 30μM C_6-dihydroceramide, 5μM *threo*-PPMP, or 5μM *erythro*-PPMP for 12h. Treatment with *threo*- and *erythro*-PPMP required 3d pre-incubation in addition to 12h treatment. Phosphatase activity was determined using the phosphorylase-a phosphatase assay. Activity is presented as percent of control of neurons maintained with NGF exclusively. Results are mean ± S.D from three independent experiments of samples processed in duplicates. Statistically significant vs. NGF as measured by the students t-test ($p<0.001$).

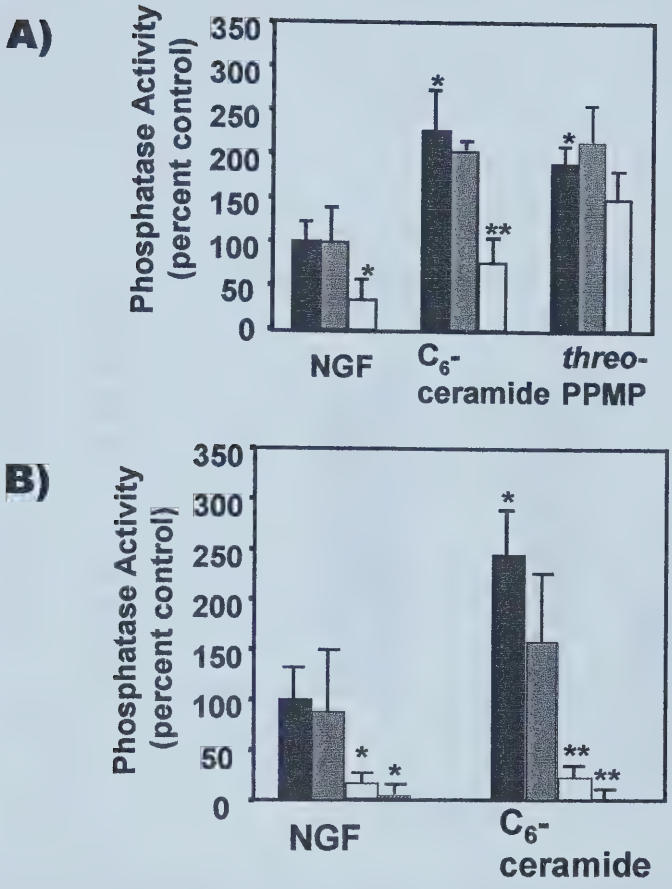

Fig.8 Ceramide activates protein phosphatases belonging to the type-1 family. A) Effect of protein phosphatase inhibitors *in vivo.* 7 day old neurons were treated with 30μM C_6-ceramide or 5μM *threo*-PPMP alone (black bars), or together with 10nM OA (grey bars) or 30μM PA (white bars). After 12h phosphatase activity was determined as for Fig. 7. Phosphatase activity is represented as percent of control of neurons maintained in NGF. Results are mean ± S.D. of three independent experiments. * statistically significant vs. NGF ($p<0.001$),** statistically significant vs. C_6-cer ($p<0.001$), as calculated by the students t-test. **B) Effect of protein phosphatase inhibitors *in vitro.*** 7 day old neurons were treated with 30μM C_6-ceramide for 12h. Cellular extracts were prepared and treated with or without phosphatase inhibitors for 30 min. prior to enzymatic analysis. No ihibitors (black bars), 2nM okadaic acid (grey bars), 200nM inhibitor-2 (white bars), or 2nM okadaic acid + 200nM inhibitor-2 (hatched bars). Results are mean ± S.D. of three independent experiments of samples processed in duplicate. * statically significant vs. NGF ($p<0.001$) as calculated by the students t-test. ** statistically significant vs. C_6-ceramide ($p<0.001$) as calculated by the student's t-test.

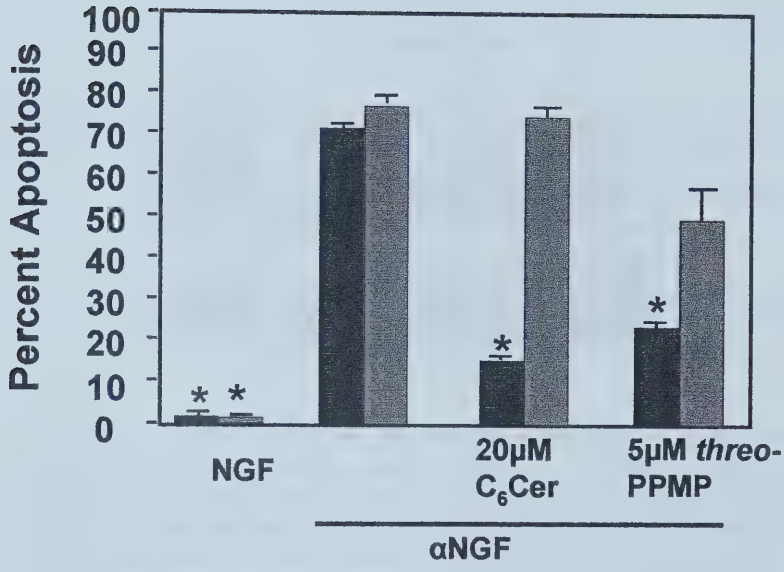

Fig. 9 - Phosphatidic acid selectively inhibits the anti-apoptotic effect of C_6-ceramide. 7 day old neurons were deprived of NGF and treated with 24nM anti-NGF antibody (αNGF), αNGF and 20μM C_6-ceramide, or αNGF and 5μM *threo*-PPMP (black bars) while some neurons received the same treatments in the presence of 30μM PA (grey bars) for 36h. Control cultures (NGF) received 50ng/ml NGF in the absence (black bars) or presence of 30μM phosphatidic acid (grey bars). Apoptosis was measured using the fluorescent mitochondrial probe MitoTracker as in Fig. 6. Results are mean ± S.D. of three experiments. Each experiment consisted of 4 wells per group and >1000 neurons were counted per treatment. * represents groups of statistical significance vs. αNGF ($p<0.001$) as calculated using the student's t-test

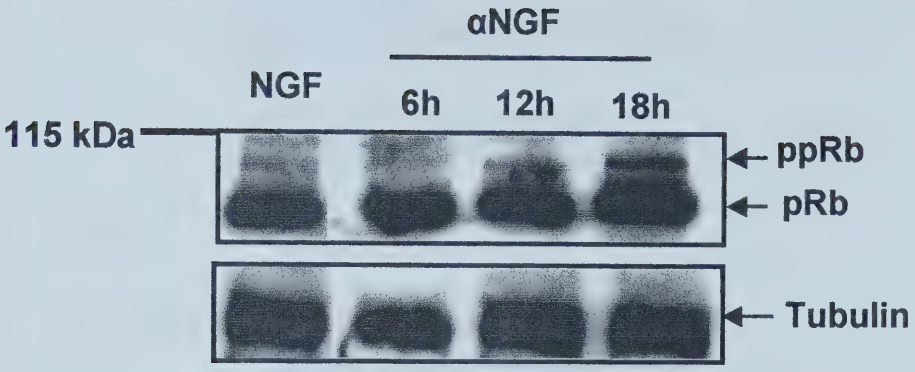

Fig. 10 - NGF-deprivation induces hyperphosphorylation of pRb. Rat sympathetic neurons were cultured in 24 well dishes in the presence of 50ng/ml NGF for 7d. After 7d neurons were depleted of NGF and treated with αNGF for the indicated times. Some neurons were maintained in 50ng/ml NGF for the duration of the experiment. At the conclusion of these times cellular material was harvested and subjected to immunoblot analysis. Antibodies to pRb and tubulin were used as indicated in Experimental Procedures. Arrows indicate bands identified as pRb. pRb denotes hypo-phosphorylated pRb and ppRb indicates hyperphosphorylated pRb. An anti-tubulin antibody was used to control for protein loading in each sample. The experiment was repeated three times. The figure depicted here is a representative blot.

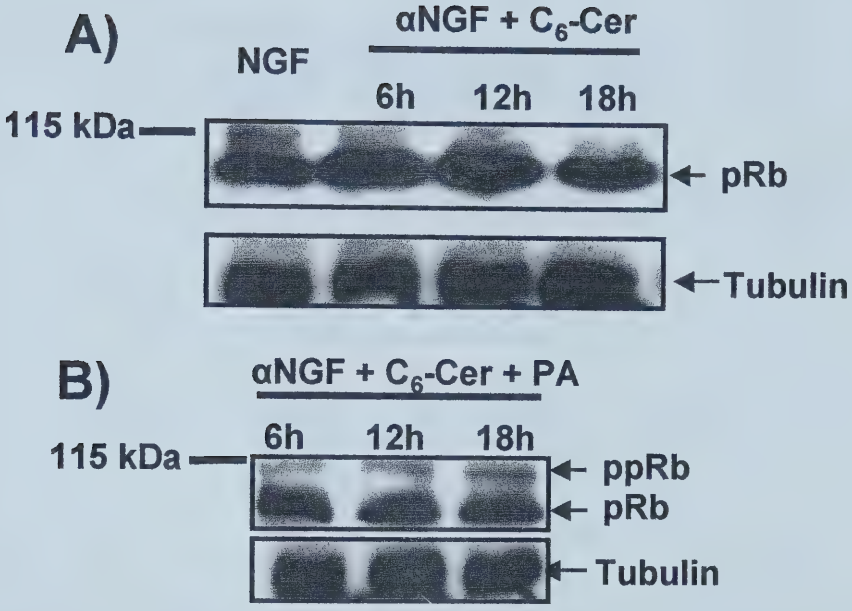

Fig 11. C_6-ceramide inhibits hyperphosphorylation of pRb in NGF-deprived neurons which can be reversed with phosphatidic acid **A)** Sympathetic neurons were cultured in 24 well dishes in the presence of 50ng/ml NGF for 7d. After 7d neurons were depleted of NGF and treated with 24nM of an anti-NGF Antibody; αNGF together with 30μM C_6-ceramide for the indicated times. Some neurons were also supplied with 50ng/ml NGF for the duration of the experiment. Following indicated times neurons were processed for SDS-PAGE and immunoblotting for pRb. Protein bands were visualized using Super Signal West Femto chemiluminescence. Top Panel; pRb immunobloting. Arrow indicates pRb band corresponding to hypophosphorylated pRb. Bottom Panel. An anti-tubulin antibody was used to measure protein content in each sample lane. Figure depicts a representative blot **B)** Neurons were cultured as in A and deprived of NGF. Neurons were treated with 24nM of an anti-NGF Antibody; αNGF together with 30μM C_6-ceramide or 30μM C_6-ceramide + 30μM PA for the indicated times. At completion of indicated time neurons were proccessed for SDS-PAGE and Immunblottin as in A. ppRb indicates hyperphosphorylated pRb and pRb indicates Hypophosphorylated pRb. Figure is a representative blot.

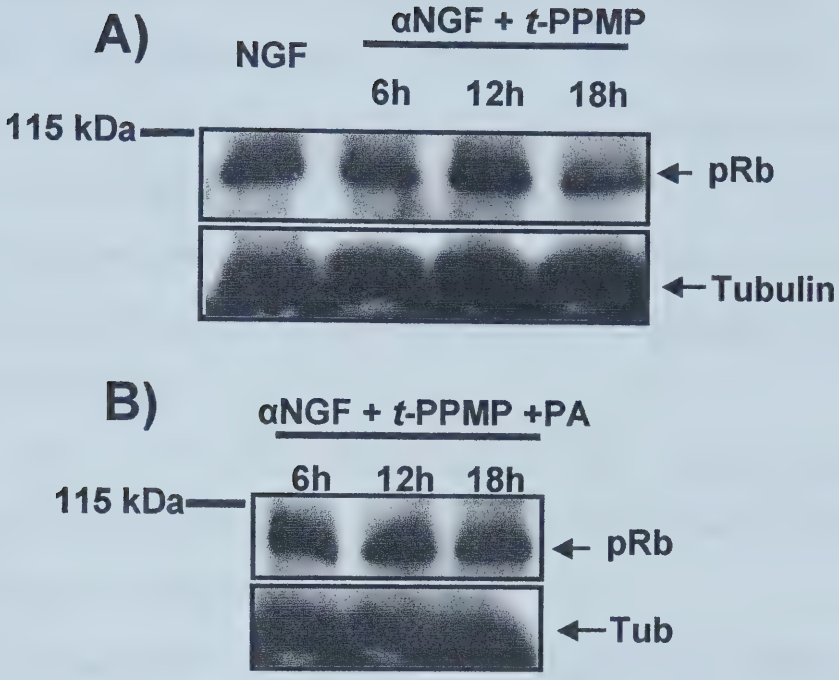

Fig 12. *Threo*-PPMP inhibits pRb hyperphosphorylation in NGF-deprived sympathetic neurons A) Sympathetic neurons were cultured in 24 well dishes in the presence of 50ng/ml NGF for 7d after which neurons were depleted of NGF and treated with 24nM of an anti-NGF antibody; αNGF together with 5μM *threo*-PPMP (*t*-PPMP) for the indicated times. Some neurons were also supplied with 50ng/ml NGF for the duration of the experiment. Following indicated times neurons were processed for SDS-PAGE and immunoblotting for pRb as in Fig.11. Arrow indicates pRb band corresponding to hypophosphorylated pRb. Bottom Panel. An anti-tubulin antibody was used to measure protein content in each sample lane. Figure depicts a representative blot **B)** Neurons were cultured as in A and deprived of NGF. Neurons were treated with 24nM of an anti-NGF Antibody; αNGF together with 5μM *threo*-PPMP (*t*-PPMP) or 5μM *threo*-PPMP + 30μM PA for the indicated times. At completion of indicated time neurons were processed for SDS-PAGE and Immunblotting as in A. pRb indicates hypophosphorylated pRb. Figure is a representative blot.

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